Germplasm Evaluation, Environmental Impact Assessment and Genetic Improvement Studies in *Jatropha curcas*

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by

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STATEMENT

I do hereby declare that the research findings of this thesis is the result of investigation carried out by me in the Centre for Energy, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Chandan Mahanta and Dr. Lingaraj Sahoo.

In keeping with general practice of reporting scientific observations, due acknowledgements have been made wherever the research findings of other researchers have been cited in this thesis.

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CERTIFICATE

This is to certify that the thesis entitled "**Germplasm Evaluation, Environmental Impact Assessment and Genetic Improvement Studies in *Jatropha curcas***", submitted by Ms. Purabi Mazumdar (07615103), a research scholar in the Centre for Energy, Indian Institute of Technology Guwahati, for the award of the degree of Doctor of Philosophy, is a record of an original research work carried out by her under our supervision and guidance. The thesis has fulfilled all requirements as per the regulations of the institute and in our opinion has reached the standard needed for submission. The results embodied in this thesis have not been submitted to any other University or Institute for the award of any degree.

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Asato maa sad-gamaya

Tamaso maa jyotir-gamaya

Mṛityor-ma-amṛitam gamaya

"Lead me from Untruth to Truth, from Darkness to Light and from Death to Immortality"

- Brhadaranyaka Upanishad — I.iii.28

Dedicated with love

To my Baba, who cherished dreams of his little daughter attaining academic excellence,

To my family, for their invaluable support and love

&

To my all favourite teachers, who brought out the best in me

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ABBREVIATIONS

ANOVA	Analysis of variance
BAP	benzylaminopurine
BA	Benzyladenine
Bt	*Bacillus thuringiensis*
CAMBIA	Centre for Application of Molecular Biology to International Agriculture
CAMV	Cauliflower Mosaic Virus
CTAB	Cetyltrimethyl ammonium bromide
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FAME	Fatty acid methyl ester
FFA	Free fatty acid
GA_3	Gibberellic Acid
GC	Gas chromatography
Gus	B-1-4 glucuronidase
GCV	Genotypic coefficient of varriation
hpt	hygromycin phosphotransferase
IBA	Indole-3- butyric acid
KOH	Potassium hydroxide
LB	Luria-Bretani
LCM	Liquid co-cultivation media
LCA	Life cycle analysis
LCIA	Life cycle impact assessment
MS	Murashige and Skoog's medium (1962)
NaOH	Sodium hydroxide
NAA	1-Naphthaleneacetic acid
NMR	Nuclear magnetic resonance
nptII	Neomycin phosphotransferase II
OD	Optical density
ORF	Open reading frame
PCR	Po lymerase chain reaction

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PCV	Phenotypic coefficient of varriation
PVDF	Po lyvinylidene fluoride
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-Polymerase chain reaction
T-DNA	Tranferred-DNA
TGA	Thermogravimetric analysis
Ti-plasmid	Tumer inducing plasmid
vir	Virulence gene

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UNITS

mm	millimetre
cm	centimetre
m^2	meter square
mg	milligram
g	gram
g/l	gram per litre
kg	kilogram
ml	mililitre
L	litre
ppm	parts per million
p	piece
MJ	mega joule
MJ/Kg	mega joule per kilogram
kWh	kilowatt hour
ha	hectare
µg	microgram
µl	microliter
µm	micromolar
mM	milimolar
pH	negative log of H^+ ion
rpm	revoloution per minute
s	second
%	percentange
°C	degree celsius
°C/min	degree centigrade per minute
v/v	Volume by volume (concentration)
w/v	weight by volume (concentration)

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ABSTRACT

The utilization of plant biofuel based *Jatropha curcas* feedstock is emerging as promising solution to problems of depletion of fossil fuel, fuel crisis and concern over global climate change. Large scale profitable cultivation of *Jatropha* is still in its infancy due to low and inconsistent yield and slow progress in identification of elite germplasm. Identification of elite germplasm from diverse agro-climatic region *vis-a-vis* development of superior genotype for higher seed yield and oil content, earlier maturity, reduced plant height, resistance to pests and diseases, drought resistance/tolerance, higher ratio of female to male flowers and improved fuel properties is expected to enhance the utility of *Jatropha* seed feedstock for biofuel. Adaptation of *Jatropha* to a wide range of climatic conditions including Northeast India, one of the biodiversity hot spot of the world, suggests existence of considerable genetic variation in growth and seed oil traits which can be potentially harnessed for selection of elite germplasm having high oil content and yield. Systematic analysis of the seed oil content and physicochemical properties forms the basis for identification of elite lines of *J. curcas* for commercial biodiesel production program. Life cycle assessment of *Jatropha* based biodiesel production can indicate its environmental impact and this analysis can also assist in adapting to appropriate biodiesel feedstock with minimum impact on environment.

Lack of disease and stress tolerant accessions of *Jatropha* prohibits its successful commercial plantation program. Introduction and expresssion of crystalline toxin genes (*cry*) derived from *Bacillus thuringiensis* (Bt) through transgenic approaches have proven as effective mechanisms for protecting crops against insect infestations. In *Jatropha*, absence of an efficient and reproducible *in vitro* regeneration system amenable to gene transfer through *Agrobacterium*-mediated transformation hinders its genetic improvement program.

In the present study, elite accessions of *J. curcas* from Assam, a state in North East India were identified on the basis of their seed trait and oil content. The oil content of the four elite accessions was found in the range of 37.6-46.6%. Analysis of physico-chemical properties of the oil and biodiesel obtained from seeds of these elite accessions demonstrated that they were within acceptable range of standards specifications of ASTM D6751. Life cycle assessment of *Jatropha* biodiesel production indicated its cultivation

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process generating highest environmental impact as compared to other stages of its life cycle, and showed higher sustainability of biodiesel from *Jatropha* as compared to *Pongamia*.

An efficient and reproducible *de novo* plant regeneration system from cotyledonary leaf segment explants amenable to genetic manipulation through *Agrobacterium*-mediated transformation was established. The choice of explants of appropriate age and the orientation of the explants in culture medium were found to exert significant influence on the frequency of *de novo* plant regeneration. Furthermore, the age of the explant was found to be the critical aspect in conferring appropriate biological condition of the explant vital for optimal infection and T-DNA transfer by *Agrobacterium tumefaciens*. Highest regeneration response was reported in the young explants, derived from the cotyledonary leaves of germinating seed compared to the leaves from one- and two-week-old seedlings. This gradient with age of the explant was observed in frequency of callus induction and shoot organogenesis from callus. The explants cultured with their abaxial side in medium showed significantly higher regeneration response. The youngest explant was found to be most amenable to *Agrobacterium*- mediated transformation as compared to older explants in recovery of stable transgenic plants in *J. curcas*. An acidic pH (5.7), addition of phenolic compound, acetosyringone at 100 µM, and co-cultivation of explants with *Agrobacterium tumefaciens* for 3 days in dark at 25°C were found critical for achieving optimal T-DNA delivery to regenerating explants. Employing these critical factors, an efficient *Agrobacterium*-mediated transformation system in *Jatropha* was established.

An insecticidal gene, *cry1Ac* was introduced to *Jatropha* and transgenic lines expressing *cry1Ac* were generated for the first time. The transgenic *Jatropha* lines with single copy of *cry1Ac* integration generated in this study will be of paramount importance for analyzing the impact of *cry1Ac* for resistance to target insects. The improved transformation system established in this study would enable transfer of a wide range of candidate genes in *Jatropha* which in turn speed up the process of *Jatropha* varietal improvement program.

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Chapter 1

General introduction

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1.1 Introduction

Limited reserve of fossil fuel, growing demand for petroleum fuel and environmental concerns due to global warming have upsurge research on alternative renewable energy sources to offset the global energy crisis. Biodiesel has become more attractive as a substitute for petroleum diesel as it is environmentally safe, renewable, non-toxic, bio-degradable with similar properties to that of petroleum-based diesel and represents four-fold higher overall positive life cycle energy balance than petroleum diesel (Kiss et al. 2006). Among the renewable feed stocks, *Jatropha* has drawn increasing attention for biodiesel production as the oil is non-edible and hence does not compromise with the production of edible oils. The *Jatropha* plant has rapid growth, higher oil content (30-60%) than other oil seed crops (Achten et al. 2008), small gestation period, adaptation to diverse environmental conditions, optimal plant size and architecture for convenient collection of seeds (Sujatha et al. 2008).

Adaptation of *Jatropha* to a wide range of climatic conditions has suggested existence of considerable genetic variation in growth, seed traits and oil content, which can be potentially harnessed for selection of elite germplasm having high oil content and yield (Kaushik et al. 2007). Northeast India, one of the biodiversity hot spots of the world, represents unique environmental conditions which support vast species diversity. *Jatropha* is well naturalized in Assam, a state in North East India. However, to our knowledge, no systematic studies has been undertaken so far to evaluate seed characteristics and oil traits of *Jatropha* germplasm of Assam for identification of elite varieties. Therefore, this research was conducted to investigate the variation in seed trait and oil content in *J. curcas* population collected from different locations of Assam.

For successful establishment of *Jatropha* as a potential biofuel feed stock, besides the oil content the evaluation of fuel properties is also necessary. Free fatty acid, acid value

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and fatty acid composition is known to show variation among *Jatropha* oils collected from various regions of the world (Achten et al. 2008). Therefore, analysis of physico-chemical properties of biodiesel is very essential to justify its suitability as alternative to diesel fuel. An effort was made to analyze physico-chemical properties of *Jatropha* oil produced from selected elite *Jatropha* accessions and compared with ASTM D6751 standard.

The production and utilization of biofuel have impacts on the environment. It is essential to evaluate their environmental impact in order to check sustainability as a potential fuel. One of the tools for evaluating environmental sustainability is life cycle assessment (LCA). Using life cycle assessment method, the impact of any product on the environment throughout the entire production phase and use processes can be evaluated (Lindfors et al. 1995). By inventorying all collected inputs of the production system and obtained output from the system, environmental performances can be calculated (Achten et al. 2008). An effort was made to investigate the environmental impact of *Jatropha* biodiesel production by using the concept of life cycle.

Contrary to popular belief that *J. curcas* are resistant to insects, it has been found to be susceptible to several groups of insects and pests during large scale plantation (Shanker and Dhyani 2006). Among the insects and pests infested on *J. curcas*, *Spodoptera litura* cause 60% to 70% damage in *Jatropha* plantation (Meshram and Joshi 1994). Spraying of commercially available insecticides is compromised with emergence of resistance biotypes (Ekesi, 1999) and environmental pollution. Therefore, genetic improvement of *Jatropha* for insect resistance can form the backbone for integrated insect pest management. Control of lepidopteran insect by *Bacillus thuringiensis* (*Bt*) spores is widely used in crop protection. However, use of Bt spore formulations is limited by its weather non-resilience nature and spores often do not certain insect pests that live within plant tissues. Therefore, genetic engineering holds great promise in developing the *Jatropha* expressing candidate genes for

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insect resistance. Overexpression of *cry1AcF* in transgenic castor through the conceptual framework of genetic transformation has been shown to confer complete resistance against target insect, *S. litura* (Kumar et al. 2011). Activated Cry1Ac toxin expressed in Bt cotton (Monsanto line NuCOTN-33B) caused an increase in mortality, a delay in development and a decrease in the body mass of first instar of *S. litura* (Zhang et al. 2006). Therefore, overexpression of *cry1Ac* in *Jatropha* is expected to confer resistance against target insect, *S. litura.*

Development of an efficient plant regeneration system amenable to genetic transformation is prerequisite for generating transgenic *Jatropha*. Although an *Agrobacterium*-mediated transformation system in *Jatropha* using cotyledonary leaf explants has been reported earlier (Li et al. 2007), the protocol was found either inefficient or difficult to reproduce, a proposition attributed to the inappropriate age of the explants used. Therefore, development of efficient regeneration system amenable to genetic transformation requires selection of explants with appropriate age and their orientation for regeneration, their amenability to *Agrobacterium*-mediated transformation and the critical parameters that affect optimal T-DNA delivery. The optimized transformation system was adopted for generation of transgenic *Jatropha* overexpressing *cry1Ac*.

The present study was undertaken with the objective of screening elite *J. curcas* germplasm from Assam, for high yield and oil content, investigating the feasibility of this oil for biodiesel production by analyzing fuel properties and environmental performance and development of transgenic *Jatropha* overexpressing *cry1Ac* for resistant to target insect, *S. litura* for stabilization of yield.

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1.2 Objectives

The present investigation was carried out with the broad objectives to screen and evaluate *Jatropha curcas* germplasm of Assam on the basis of seed trait, oil content and physicochemical characteristics and analysing the environmental impact produce by *Jatropha* biodiesel production process and establishment of an efficient genetic transformation system to generate stable transgenic *Jatropha* against target insect for stabilization of yield. The salient objectives outlined as:

- Evaluation of seed traits and oil content of *J. curcas* germplasm of Assam
- Physico-chemical analysis of biodiesel of *Jatropha* from Assam
- Life cycle assessment of *Jatropha* biodiesel production for evaluation of sustainability
- Establishment of efficient *Agrobacterium* mediated transformation in *J. curcas*
- Generation and analysis of transgenic *Jatropha* overexpressing cry1Ac gene

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Chapter 2

Literature review

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2.1 *Jatropha curcas* - a promising non-edible source of biodiesel

Increase in crude oil prices, depleting fossil fuel and global climate change have upsurged research on development of renewable and alternate sources of energy such as biofuel. The utilization of energy crops as a source of biofuel is gaining importance in developing countries due to the heavy burden on economy for crude oil import, uncertainty in crude oil supply from oil reserve countries with political unrest, pressure to reduce emissions of greenhouse gases and availability of huge land resources for energy crop plantation. Among the energy crops, oil seed bearing plant, *Jatropha* (*Jatropha curcas*) is being promoted as the source of biofuel due to the relatively high seed oil content (30%–60%) and physicochemical properties of oil similar to that of fossil fuel (Pant et al. 2006; Kaushik et al. 2007; Akbar et al. 2009; Supamathanon et al. 2011) . Its non-edible biodiesel feedstock minimizes the competition with edible oil supply. This crop has potential to grow on degraded and marginal lands, which are not otherwise favorable for agricultural use (Francis et al. 2005; Jongschaap et al. 2007; Jain et al. 2010). Biodiesel prepared from *Jatropha* oil has a good oxidation stability compared to soybean oil, low viscosity compared to castor oil and a low pour point compared to palm oil (Li et al. 2012). It emits 80% less CO_2, 100% lower SO_2, and has a higher flash point than diesel fuel (Nahar et al. 2011). The fuel properties of *Jatropha* based biodiesel have been found close to those of fossil diesel and match the American and European standards (Yang et al. 2012).

Despite its numerous advantages, the full potential of *J. curcas* has not been realized primarily due to the lack of uniformity in seed and oil yields and low economic returns (Achten et al. 2008). Limited information available on genetic variability, genotype environmental interaction and agronomy of *Jatropha*, high male to female flower ratio, asynchronous flowering and susceptibility to diseases and yield limiting abiotic stresses are the other major factors limiting the prospects of *J. curcas* as a potential biofuel crop (Kaushik et al. 2007; King et al. 2009; Parthiban et al. 2009;

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Divakara et al. 2010; Alam et al. 2011). Intensive efforts are being made for an in breadth genetic diversity assessment involving physicochemical characterization and use of molecular markers, large-scale transcriptome and proteome analysis, identification of candidate genes for trait improvement, whole genome sequencing (Johnson et al. 2011). Along with that, development of efficient transformation system for incorporation of candidate genes of oil improvement, resistance to biotic and abiotic stresses and manipulation of female to male flower ratio are the approaches initiated for improvement of this biofuel crop. Keeping these in view, this chapter attempts to present the current status of knowledge vis a vis the integrated approach aimed at selection of elite germplasm and genetic improvement of *Jatropha*.

2.2 Taxonomy and botanical description of *Jatropha curcas*

The genus *Jatropha* belongs to tribe Joannesieae of Crotonoideae in the Euphorbiaceae family and contains approximately 175 known species. Linnaeus (1753) was the first to name the physic nut as *Jatropha curcas* L. in "Species Plantarum". The genus name *Jatropha* derives from the Greek word jatrós (doctor) and trophé (food), which implies its medicinal uses (Kumar et al. 2008). *Jatropha* is native to Central America but is now found abundantly in many tropical and sub-tropical regions of Asia and Africa (Heller, 1996). Dehgan and Webster (1979) revised the subdivision made by Pax (1910) and now distinguish two subgenera (Curcas and *Jatropha*) of the genus *Jatropha*, with 10 sections and 10 subsections to accommodate the Old and New World species. They postulated the physic nut {*J. curcas* L. [sect. Curcas (Adans.) Griseb., subg. Curcas (Adans.) Pax]} to be the most primitive form of the *Jatropha* genus, which form artificial and natural hybrid complexes readily and poses a problem to the genetic fidelity (Prabakaran and Sujatha 1999). Species in other sections evolved from the physic nut or another ancestral form, with changes in growth habit and flower structures.

Jatropha is a small tree or large shrub, which normally reaches a height of three to five meters, but under favourable conditions can attain a height of 8 to 10 m. The

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plant shows articulated growth (Kumar et al. 2008), straight trunk, thick branchlets with a soft wood and a life expectancy up to 50 years (Achten et al. 2008). Normally, five roots are formed from seedlings, one central and four peripheral (Kumar et al. 2008). The trees are deciduous and flowering occurs during the wet season (Raju et al. 2002), often with two flowering peaks, i.e. during summer and autumn (Divakara et al. 2010). The plant is monoecious and flowers are unisexual; occasionally hermaphrodite flowers appear (Dehgan and Webster 1979) (Fig. 2.1. a, b, c). The inflorescence is axillary paniculate polychasial cymes formed terminally and the unisexual male flowers contain 10 stamens arranged in 2 distinct whorls of 5 each while the female flowers is tricarpellary, syncarpous with trilocular ovary dilating to a massive bifurcate stigma (Dehgan and Webster 1979). *Jatropha* is self-compatible (Heller et al. 1996), but cross-pollination is supported by a time gap between anthesis of male and female flowers (Heller, 1992).

Fig. 2.1 ***Jatropha curcas* plant** (a) Young *Jatropha* plant with flowers (b) Axillary paniculate polychasial cyme inflorescences of *J. curcas*. (c) Inflorescences of *J. curcas* containing both male staminate flowers (M) and female pistillate flowers (F) (d) *Jatropha* plant bearing fruits

The existence of protandry in *J. curcas* provides time for receptive stigma to get pollens from male flowers, and enhance the opportunity of reproductive success

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(Divakara et al. 2010). However, in some racemes, the female flowers open readily showing a tendency to promote xenogamy and minimize geitonogamy (Chang-wei et al. 2007). Mostly flowers are pollinated by insects especially honey bees. In good rainfall conditions, nursery plants may bear fruits after the first rainy season and directly sown plants after the second rainy season (Kumar et al. 2008).

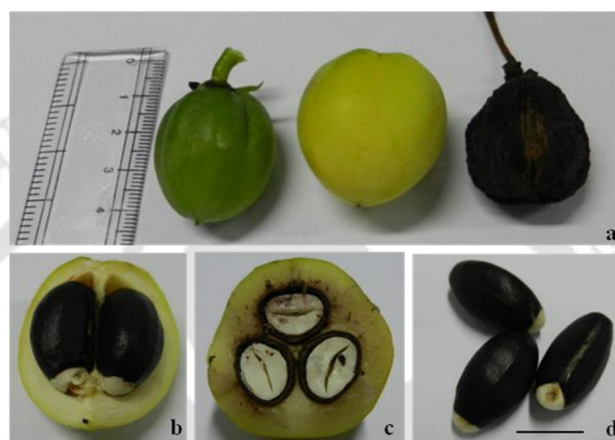

Fig. 2.2 Fruit of *J. curcas* (a) in three developing stage (b) Seed inside fruit (c) Cross-section of a *J. curcas* seed pod containing three developing seeds (d) Mature seeds of *J. curcas* (bar:1cm)

Each inflorescence yields a bunch of approximately 10 or more ovoid fruits (Tewari et al. 2007). The fruit is an ellipsoid capsule green in colour, turns yellow when matures and finally turns black that contains 2-3 black seeds per fruit (Fig. 1d, 2a, 2b, 2c). Seeds are ellipsoid, triangular-convex in shape (Fig. 2.2 d).

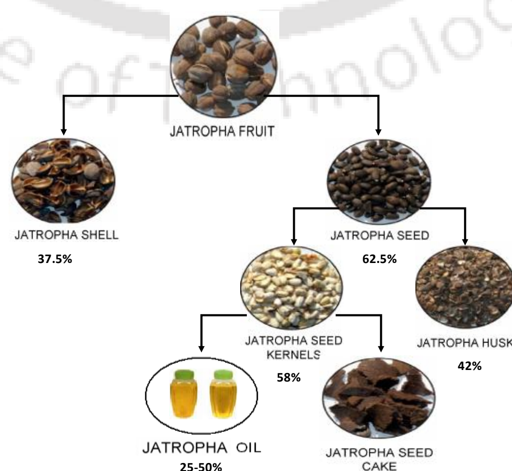

Fig. 2.3 **Composition of *J. curcas* Fruit** (Source: Abreu, 2009)

Fruit is made up of 37.5% shell and 62.5% seed, whereas, seed consists of 42% husk and 58% kernel (Abreu, 2009) (Fig. 2.3). The seeds contain 25- 50% oil with 21% saturated fatty acids and 79% unsaturated fatty acids (Gubitz et al. 1999). Curcin and phorbol ester are the two toxic compounds present in seed endosperm which makes *Jatropha* oil non-edible (Islam et al. 2011). *Jatropha* is a diploidspecies with 2n = 22 chromosomes. *G*enome size is 416 Mbp and the karyotype is made up of 22 relatively small sized chromosomes (1.71 to 1.24 mm) (Carvalho et al. 2008)

2.3 Distribution and ecological adaptability of the crop

Jatropha was probably distributed by the Portuguese via the Cape Verde Islands and Guinea Bissau to other countries in Africa and Asia (Heller, 1996). It is classified as the climax vegetation of tropical savannas in the dry (Godin et al. 1971) or semi-dry tropics (Martin et al. 1983). It grows under a wide range of rainfall regimes from 250 to over 1200 mm per annum (Katwal and Soni 2003). Its drought tolerance and adaptation capacity is extraordinarily high. *Jatropha* have been reported to grow even where there is no rainfall for 2– 3 years (Munch and Kiefer 1981). Normally optimum growth temperature for this crop is around 25–35°C, but it can also adapt and grow in higher altitudes with the risk of light frost (Makkar et al. 2009). It can tolerate temperatures far above 40°C, but temperatures below 20°C for a week or even shorter periods initiate leaf shedding (Makkar et al. 2009). This plant grows almost anywhere except waterlogged lands, even on gravelly, sandy and saline soils (Kumar et al. 2008). This crop makes symbiotic relationship with arbuscular mycorrhizal (AM) fungi present in the soil (Singh et al. 2010), which provides essential nutrients like P, Cu, Zn etc, which are often present in low concentration in soil (Liu et al. 2007). It sheds leaves during winter season, which form mulch around the plant base. The formation of mulch increases organic matter in soil which helps to enhance earthworm population around the root zone, which finally leads to increasing soil fertility (Singh et al. 2010),

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representing *Jatropha* as a suitable species for soil conservation and reclamation (Frienvus, 2008).

2.4 Potential of *J. curcas* products

Jatropha has immense economic potential and environmental significance. The crop has diverse uses ranging from biodiesel production for fossil fuel replacement, traditional medicine for common human and animal ailments; protection against land erosion, boundary fence or live hedge to production of valuable product in chemical and cosmetic industries (Openshaw, 2000). Uses of various parts of *Jatropha* plant is presented in Fig 2. 4.

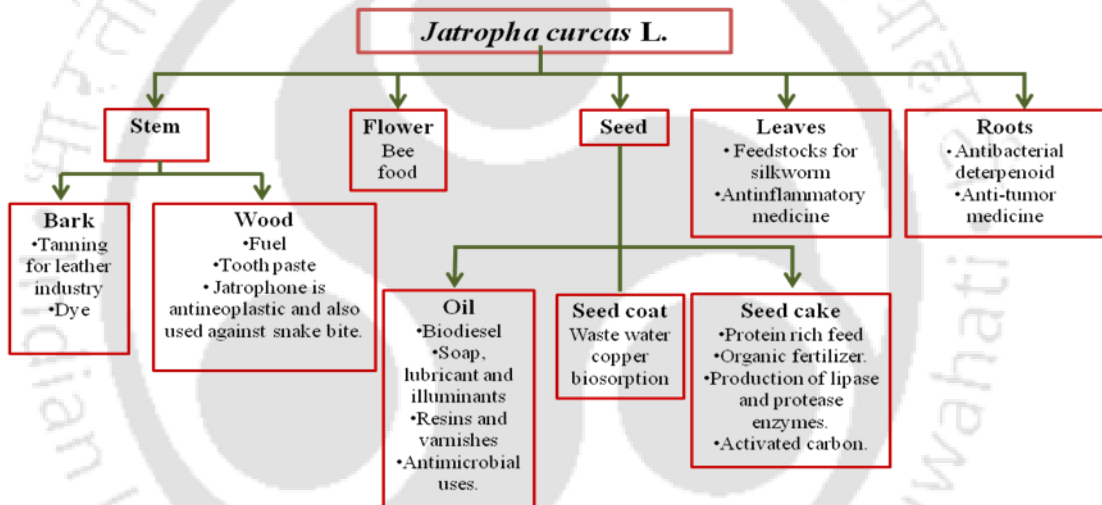

Fig. 2.4 **Uses of various parts of *Jatropha curcas*** (Source: Adapted from Heller, 1996; Openshaw, 2000; Shrivastava et al. 2008; Burkill, 1985; Biehl and Hecker 1986; Brum et al. 2006; Roy, 1998; Eshilokun et al. 2007; Patel et al. 2008; Jain et al. 2008; Devappa et al. 2008; Mendoza et al. 2007; Mahanta et al. 2008; Sricharoenchaikul et al. 2008; Alyelaagbe et al. 2007)

2.5 Genetic variation studies

Diverse agro ecological regions and climatic conditions offer a good opportunity for an exotic species to generate variation. *Jatropha* being an exotic species is well naturalized among the flora of diverse landforms of India, so there is high probability of existence of considerable amount of genetic variation. It has been observed that physical characteristics of *J. curcas* seeds, varies depending on their geographical origin and climatic condition. Generally, seed weight varies from 0.4 to 0.7 g and seed dimensions

vary with length and width from 15-17 mm and 7-10 mm, respectively (Martinez-Herrera et al. 2006). The kernel to shell ratio is usually around 60:40 (Makkar et al. 1997). Oil content of seed is in the range of 30%-42% (Surwenshi et al. 2011) (w/w). This variation clearly indicates the prominent role of habitat and prevailing environmental conditions over genotype in determining phenotypic variations in *J. curcas* (Kaushik et al. 2007). Collection and selection of elite genotypes are of importance to understand degree of genetic variation in native populations of *J. curcas* (Openshaw, 2000; Ovando-Medina et al. 2009; Sujatha et al. 2005). Some varieties of *J. curcas* recognized in the world based upon their phenotypic traits or the toxicity of seed, but this classification is not realistic, factor of arbitrariness is more. For example, three varieties, Cape Verde variety that has spread all over the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety that has only traces of phorbol esters in the fruit (Heller, 1996; Henning, 1997; Sujatha et al. 2005). Recently, some commercial varieties have been released, e.g. SDAUJ1, from an Indian program of selection of germplasm (Basha and Sujatha 2007) and JMAX, derived of Guatemalan germplasm (www.sgbiofuels.com). However, comparative studies of such varieties grown in the same site have not been reported. In addition, there is limited information with regard to the number of introductions and the genetic diversity of *J. curcas* populations grown in different parts of the tropics. Sakaguchi and Somabhi (1987) conducted a survey on 40 clonal lines of *J. curcas*, obtained from different locations in Thailand. However, no significant intraspecific morphological variations were observed among the forty clonal lines of *J. curcas*. Rafii et al (2012) found no significant variation among the accessions collected from peninsular Malaysia except in plant height, seed thickness, seed breadth, and total seed per accession. They concluded that environmental factors played an important role than the genetic factor. Comprehensive work on collection of germplasm and evaluation of growth, morphology, seed characteristics and yield traits is still in its nascent stage in India. Release of high

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yielding cultivars is impossible without ascertaining the magnitude of variation present in the available germplasm, interdependence of growth pattern with yield, extent of environmental influence on these factors, heritability and genetic gain of the material. The knowledge on variation of this kind would be useful for genetic selection especially in a crop where the desired ideo type is yet to be defined due to its wide adaptability and end use pattern. Knowledge of genetic variation, pest resistance, drought hardiness and yield attributes in wild germplasm of *Jatropha* can be of great potential in its improvement programmes, particularly selection of genotypes having more oil content and yield. On contrary to the previous reports, significant variability has been observed in Indian accessions of *J. curcas* in terms of morphology, germination and seedling growth in central India (Ginwal et al. 2005). They observed high percentage of heritability coupled with moderate intensity of genetic gain for seed germination traits, signifying that germination is under strong genetic control and good amount of heritable additive genetic component can be exploited for improvement of this species (Ginwal et al. 2005). Considerable variation has been also reported in the seed characteristics and oil content of *J. curcas* in 24 accessions collected from different zones of Haryana state, India (Kaushik et al. 2007). There were significant differences observed ($P<0.05$) in seed size, 100-seed weight and oil content between accessions. However, the coefficient of variation was higher for phenotype than genotype, indicating a predominant role of the environment. Sunil et al. (2008) and Mishra (2009) reported similar results while selection of candidate plus accessions of *J. curcas* from India, correlating morphological characteristics (plant height, collar height and thickness, number of primary branches, petiole length, number of fruits per cluster, pedicel length and seed yield) with the oil content of the seed. Rao et al (2008) reported considerable genetic variation in growth, chemical composition of seed and seed traits (seed size, 100 seed weight, oil content, plant height, no of branches, flower, female to male flower ratio, flowering and fruiting time and seed yield) at the level of provenance, variety or progeny in *J. curcas*

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population from different locations in Andhra Pradesh, India. Gohil et al (2008) studied genetic variability of seven Indian accessions from Gujarat and two nontoxic accessions from Orissa and nine accessions from states of Rajasthan and Gujarat (Gohil et al. 2009). They analyzed fourteen characters (plant height, basal height, canopy size, collar diameter, number of leaf, capsule and seed, number of primary, secondary and tertiary branches, average seed weight and oil content) in accessions and observed moderate genetic diversity and none of the characters showed heritability over 75% indicating the role of environment. Mohapatra et al (2010) observed significant variation in growth, phenology and seed characteristics in twenty randomly selected seeds of *Jatropha* collected from different agroclimatic zones of India in a progeny trial under tropical monsoon climatic conditions of Bhubaneswar, Orissa. They found significant correlation between the length of branch, number of inflorescence and number of fruits in plant. Wani et al (2012) evaluated seven accessions from North India (Jammu and Kashmir state) on the basis of plant height and spread, stem diameter, leaf length and width, seed yield and 100 seed weight, oil content and chemical composition of seed to identify elite accession for commercial cultivation.

North east India is considered as one of the biodiversity hotspots of India. *Jatropha* is found in diverse landform of Assam, a state of North East India. The government has also started large scale plantation in Assam, without proper study on screening and identification of *J. curcas* from North East India for seed trait and high oil content. Saikia et al (2009) carried out variability study that included four accessions from Assam and eight accessions from other states of North East India along with other accessions from India. They analyzed genetic variation on the basis of plant height, stem girth, branches per plant and seed weight. But they did not consider seed traits and oil content, which are most important attributes for evaluation of any biofuel crop and for selecting elite germplasm for commercial cultivation in that region.

2.6 Seed oil of *J. curcas*

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Seeds are the source of oil of *Jatropha*. Each mature tree on average produces about four kilograms of seed per year when cultivated under optimal condition (Banapurmath et al. 2008; Tamalampundi et al. 2008). Mature *Jatropha* plantations yield 4 to 5 tonnes of seed per hectare with a long productive period of around 30 - 50 years, which equate to approximately 1.5 tonnes of oil per hectare (Matsuno et al. 1984; Foidl et al. 1996). Oil is extracted from seeds mechanically, chemically and enzymatically.

2.6.1 Mechanical extraction

Oil presses have been used for extraction of *Jatropha* oil, as simple mechanical devices either powered or manually driven. The most commonly used oil presses are ram press (Forson et al. 2004) or driven screw press (Foidl et al. 1996). For mechanical extraction, seeds were first dried properly, dehulled and then pressed. The resulting oil has to be filtered to remove sludge. Engine driven screw press extracts more oil compared to Ram press (Henning et al. 2000).

2.6.2 Chemical extraction

Solvent Extraction is a chemical process which involves extracting oil from seeds by treating it with an organic solvent having low boiling point as opposed to extracting the oils by mechanical pressing. Solvent extraction is the most widely used technique, owing to their high efficiency in oil recovery (90% to 98%). The solvent extraction method recovers almost all the oils and leaves behind only 0.5% to 0.7% residual oil in the raw material, whereas in mechanical pressing the residual oil left in the oil cake from 6% to 14%. Hexane having low boiling point (67°C / 152°F), high oil solubility and easy recovery property, is considered as one of the best solvents for extraction process as compared to petroleum ether and isopropanol (Patel et al. 2011). The extraction process consists of treating the raw material with hexane and recovering the oil by distillation. The left out hexane can be reused after extraction.

2.6.3 Enzymatic extraction

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Aqueous enzymatic oil extraction (AEOE) has emerged as promising technique for oil extraction from plant material (Patel et al. 2011). The enzymes used in extraction enhance oil recovery by breaking cell walls and oil bodies. This process is eco-friendly and does not produce volatile organic compounds as atmospheric pollutants (Rosenthal et al. 1996). The major disadvantage associated with AEOE is the long process time necessary for the enzymes to liberate oil bodies. Another factor is the commercial nonavailability of enzymes which are used in extraction (Shah et al. 2005) and the process is costly. This prevents the use of the process by other researcher. Shah et al. (2005) reported combination of ultrasonication and aqueous enzymatic method to extract oil from *J. curcas* seed kernels. The maximum yield of 74% was obtained by ultrasonication for 5 min followed by aqueous enzymatic oil extraction using an alkaline protease at pH 9.0. Use of ultrasonication also resulted in reducing the process time from 18 to 6 hour.

Enzyme assisted three phase partitioning methods are also employed for oil extraction from *Jatropha*. This process consisted of simultaneous addition of t-butanol (1:1, v/v) and 30% (w/v) ammonium sulphate to the slurry prepared from *Jatropha* seed kernels. Combination of sonication and enzyme treatment with a commercial preparation of fungal proteases at pH 9, led to 97% oil yield within 2 h. The advantage of this method is unlike the soxhlet extraction, which is carried out for 8-24 h whereas it takes only about 2 h (Shah et al. 2004a).

2.7 Biodiesel production

This seed oil of *Jatropha* is easily convertible to biodiesel.

Fig. 2.5. **Transesterification reaction of oil**, where, R1, R2 and R3 represent the different fatty acid derivatives (long unbranched aliphatic tail (chain), which are either saturated or unsaturated)

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Biodiesel is monoalkyl esters of fatty acid prepared by transesterification of oil. The transesterification reaction consists of transforming triglycerides into fatty acid alkyl esters in presence of catalyst (Palligarnai et al. 2008) (Fig. 2.5). This process consisted of three consecutive reversible reactions, where in, oil was successively converted into diglyceride and monoglyceride, and then into glycerine and FAMEs (fatty acid methyl esters). Two approaches are followed for transesterification; first one is chemical, where, alcoholysis of oil is performed by short chain alcohol and second one is enzymatic approach, in which lipase –catalyzed transesterification is performed in nonaqueous environments (Shah et al. 2004b). Chemical approaches are superior in terms of reaction time and cost, whereas, enzyme catalyst one is superior in terms of separation process of glycerol. Transesterification reaction is quite sensitive to various parameters which decide the reaction completion and yield to a significant extent (Sharma et al. 2009). These parameters include free fatty acids (FFAs), molar ratio of alcohol to oil, catalyst, reaction temperature, time and stirring. Table 2.1, shows the work carried out for optimization of *Jatropha* biodiesel production using different approaches.

2.7.1 Chemical approaches

Chemical approaches for transesterification reaction are catalytic and non-catalytic transesterification (Demirbas et al. 2005) and other methods such as supercritical processes, microwave and ultrasound systems (Demirbas et al. 2009; Barnard et al. 2007; Santos et al. 2009). Transesterification reaction can be catalyzed by both homogeneous (alkalis and acids) and heterogeneous catalysts. Homogeneous catalysts are highly efficient and take very less reaction time in transesterification of oil to obtain bio-diesel. However, this catalyst shows greater performance only when the free fatty acid content of oil is less than 1% (Karmakar et al. 2010). The main drawback of homogeneous catalysts is energy intensive separation of glycerol and soap from biodiesel, when free fatty acid (FFA) content is more (Berchmans et al. 2008).

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Heterogeneous catalysts are promising for the transesterification reaction of vegetable oils containing high FFA (>1%) and also biodiesel can be easily separated from by-products. Heterogeneous catalysts are environmentally benign, could be operated in continuous processes and more over they can be reused and regenerated (Borugadda et al. 2012).

A. Alkali catalyst

Alkali-catalyzed transesterification process is relatively much faster compared to acid catalyzed reaction. But, the efficiency of the process is greatly governed by FFA content of oils. If the FFA content of oil is more, it reacts with base catalyst to form soap and water. Formation of soap not only lowers the yield of alkyl esters but also increases the difficulty in the separation of biodiesel from by-products. Therefore, for high FFA oil feedstock's (> 1), a two-stage transesterification process usually followed to reduce the FFA content in oil. After acid pretreatment or acid catalyst esterification subsequent base catalyzed transesterification improves the methyl ester yield (Berchmans et al. 2008). Among the base catalyst, methoxide catalysts shows better yields than hydroxide catalysts, and potassium-based catalysts shows higher production rate than sodium-based catalysts (Shahid and Jamal 2008).

a. Homogeneous alkali-catalyzed transesterification

Homogeneous alkali catalysts which mainly used in transesterification of vegetable oils are KOH, NaOH and CH_3ONa (Mangesh et al. 2006). These catalysts are highly promising for their industrial application because of cheap price, easy availability and competency to catalyze the reaction at low temperature and atmospheric pressure which leads to high conversion in a minimal time (Edgar et al. 2005). The major concern in using alkali catalysts is the FFA content of feedstocks. This type of catalyst is only suitable for transesterification of vegetable oil having acid value less than 2 mg KOH/g (Yong et al. 2006). A high acid value or FFA content enhances soap formation and the separation of end products becomes hard, resulting in low yield of biodiesel (Berchmans et al. 2008).

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Therefore, the pretreatment is necessary for processing oil having high FFA content. Transesterification of *Jatropha* oil using homogeneous alkali catalyst method has been found to be widely used (Foidl et al. 1996; deOliveira et al. 2009; Raja et al. 2011; Chitra et al. 2005)

b. Heterogeneous alkali-catalyzed transesterification

Heterogeneous alkali catalysts which are mainly used for transesterification of oils are basic zeolites, alkaline earthmetal oxides and hydrotalcites (Zabeti et al. 2009). Homogeneous alkali catalyst cannot be recovered after the transesterification is over; it neutralized in washing step with water and disposed as a waste product. A heterogeneously-catalyzed offers advantages over homogeneous catalysts, because of their reusability, less corrosive nature and economization of the catalyst removal step. Vyas et al. (2009) studied transesterification of *Jatropha* oil with methanol using heterogeneous alkali catalyst, alumina loaded with potassium nitrate. With this solid alkali catalyst of 6% (w), a molar ratio of methanol to *Jatropha* oil of 12:1 and a reaction time of 6 h, they are able to achieve a biodiesel yield over 84% at 70°C. However, in comparison to homogeneous alkali catalyzed transesterification, the heterogeneous alkali catalyst mediated transesterification required a higher molar ratio of methanol to oil, higher catalyst concentration and more reaction time for conversion to biodiesel. Therefore a heterogeneous alkali catalyst is less attractive to be implemented on an industrial scale for biodiesel production due to its lower catalyst activity as well as efficiency (Koh et al. 2011).

There are few reports on transesterification of *Jatropha* oil using heterogeneous alkali catalyst (Table 2.1). Payawan et al (2010) studied transesterification of *Jatropha* oil of high acid value (107 mgKOH/g) using amino-functionalized Zeolite Y, amino-functionalized MCM-41 and TBD-functionalized MCM-41 soild alkali catalyst (Table 2.1). Transesterification using 10% by weight of these developed catalysts and methanol-oil molar ratio of 15:1 at 80°C for two hours, the authors achieved biodiesel yield of

86.60%, 74.94% and 81.86%, respectively. Deng et al (2011) produced biodiesel from *Jatropha* oil using solid basic catalyst Mg/Al oxides and recorded biodiesel yield upto 95.2%, and the biodiesel properties were found close to those of the German standard. Highina et al (2012) used zinc oxide as catalyst for production biodiesel from *J. curcas* oil in a batch reactor and 98% methyl ester yield was obtained (Table 2.1).

B. Acid catalyst

Acid catalyst (H_2SO_4, H_3PO_4,) are used to convert FFAs to esters as a pretreatment step for oil feedstock containing high FFA and these are characterized by slow reaction rate and requirement of high ratio of alcohol (Gerpen et al. 2004). Efficiency of acid catalyzed reaction can be improved by increasing the molar ratio of alcohol to oil, reaction temperature, catalyst concentration and the reaction time (Gerpen et al. 2004; Mangesh et al. 2006).

a. Homogeneous acid-catalyzed transesterification

Homogeneous acid catalysts mainly used in transesterification of vegetable oils are H_2SO_4, HCl, BF_3, H_3PO_4, and organic sulfonic acids (Georgogianni et al. 2009). The advantage of the acid catalyst is that it is not strongly affected by the presence of FFAs in the feedstock. These types of catalysts can directly produce biodiesel from low-cost lipid feedstock with FFA content greater than 6% (Vyas et al. 2009). Acid catalytic transesterification of vegetable oils has been studied exhaustively (Freedman et al. 1984; Miao et al. 2009). Although, this process is insensitive to FFAs in the feedstock, homogeneous acid-catalyzed transesterification has been largely ignored mainly due to slower reaction rate, high methanol to oil ratio and higher process temperature than the base-catalysed reaction (Georgogianni et al. 2009). To our knowledge, only a single study has been reported on acid-catalysed transesterification of *J. curcas* oil. Shuit et al. (2009) studied the transesterification of *Jatropha* oil with FFA 15% to produce biodiesel by using concentrated sulphuric acid as catalyst (Table 2.1). About 99.8% of *Jatropha*

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methyl ester was obtained using 15wt% of H_2SO_4 and methanol to seed ratio 7.5 ml/g, at 60°C, for a reaction period of 24 h.

b. Heterogeneous acid-catalyzed transesterification

Heterogeneous acid catalysts have the potential to replace strong liquid acids to eliminate the corrosion problems and consequent environmental hazards posed by the homogeneous acid catalysts (Helwani et al. 2009). Heterogeneous acid catalysts which are mainly used in transesterification of vegetable oils are tungstated zirconia–alumina (WZA), sulphated zirconia–alumina (SZA) and sulfated tin oxide (STO) (Satoshi et al. 2004). These types of catalysts are tolerant to high FFA content, esterification and transesterification occur simultaneously, eliminating the washing step of biodiesel. Recycling of catalyst is possible and it reduces corrosion (Mangesh et al. 2006; Jitputti et al. 2006; Suarez et al. 2007). The use of solid acid catalysts for transesterification reaction is limited due to the low reaction rates and adverse side reaction as the factors governing the reactivity of solid catalysts have been poorly understood (Helwani et al. 2009).

In case of *Jatropha* biodiesel production, solid acid-catalysed transesterification is hardly performed or proposed by other researchers as an alkali-catalyst is considered as the most favourable (Koh et al. 2011). Feng et al. (2008) have reported preparation of *Jatropha* biodiesel using solid acid catalysts ZrO_2-$SO4_2$- and $K_4Zn_4[Fe(CN)_6]_3$ (Table 2.1). They compared the catalytic activity and stability of both the catalyst in varied acid value and reaction temperature. The results showed that the ZrO2-SO42- lost its catalytic activity in high acid value and methyl ester yield was up to 84%, whereas, K4Zn4[Fe(CN)6]3 remained its good catalytic activity and stability and yield of methyl ester up to 93%.

2.7.2 Enzymatic approaches

Enzymatic transesterification is continued to draw the attention of researchers due to the easy product separation, minimal wastewater treatment, easy glycerol separation,

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absence of side reactions and more environmentally friendly (Mangesh et al. 2006; Raman et al. 2008). The realistic problem is enzymes are expensive and its regeneration and reuse are limited with requirement of long operating time. The reaction yields from enzyme-catalysed transesterification are still unfavourable compared to the base-catalysed reactions and thus render the process impractical and uneconomical (Demirbas et al. 2005; Miao et al. 2009). In order to overcome these problems, several potential solutions have been introduced in the last few years, which include the use of immobilized lipases, several types of solvents, acyl acceptors and substrate oil sources (Borugadda et al. 2012). Several works has been carried out on lipase-catalysed biodiesel production in *Jatropha*. Shah et al. (2004b) screened different lipases (*Chromobacterium viscosum*, *Candida rugosa*, and *Porcine pancreas*) for a transesterification reaction of *Jatropha* oil in a solvent-free system to produce biodiesel (Table 2.1). Among them only lipase from *Chromobacterium viscosum* was found to give appreciable yield. Immobilization of lipase (*Chromobacterium viscosum*) on Celite-545 enhanced the biodiesel yield from 62% to 71% by using free tuned enzyme preparation with a process time of 8h at 40°C. Kumari et al (2009) have studied transesterification of *Jatropha* oil using immobilized lipase from *Enterobacter aerogenes* and obtained 94% yield of biodiesel after 48 h of reaction time. Immobilized lipases were used to facilitate its recovery after the reaction but the complexity of the lipase purification process has restricted its use in industrial applications (Koh et al. 2011). To overcome this, Tamalampudi et al. (2008) recommended the production of biodiesel from *Jatropha* oil using the whole cell of lipase producing *Rhizopus oryzae* (ROL) immobilized onto biomass support particles. The methyl ester yield achieved was 80% after 60 h using 4% of ROL (by weight of oil) in the presence of 5% (v/v) added water, methanol to oil ratio of 1:1 and reaction temperature of 30°C. Furthermore, it was found economical and practical as the lipase exhibited more than 90% of its initial activities after 5 repeated cycles of usage.

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Table 2.1 Method used for production of biodiesel from *Jatropha* oil

Transesterification	Catalyst	Catalyst amount (%)	Alcohol	Oil to alcohol molar ratio	Optimum reaction condition	FAME yield (%)	Reference
Chemical approach	KOH	1.6	Methanol	2.3:10.34	30°C, 5 h,	92	Foidl et al. 1996
Base catalysed		3.0	Ethanol	1.14: 6.9	75°C, 90 min	88.4	
	KOH	1.3	Methanol	1:6	90°C, 2 h	>97	Lu et al. 2009
	KOH	1	Methanol	1:6	60±0.3°C, 30 min, 600 rpm	84.2	Wang et al. 2010
	NaOH	1.0	Methanol	1:6	65°C, 120 min, 600 rpm	-	Rashid et al. 2010
	NaOH	0.7	Methanol	1:6	65°C, 2 h	97	Singh et al. 2009
	NaOH	1.1	Methanol	1:7	60°C, 120 min	98	Qian et al. 2010
	NaOH	1.4	Methanol	1:4.1	65°C, 2 h, 400 rpm	90	Berchmans et al. 2008
	NaOH	1	Methanol	1:5	60°C, 90 min	98	Chitra et al. 2005
	NaOH	1	Methanol	1:6	60°C, 40 min	98.6	Nakpong et al. 2010
	NaOH	0.8	Methanol	1:5	65°C, 1h	95.5	Ojolo et al. 2011
	KNO3/Al2O3	6	Methanol	1:12	70°C, 6 h	>84	Vyas et al. 2009
	amino-functionalized Zeolite Y					86.6	
		10	Methanol	15:1	80ºC, 2 h		Payawan et al. 2010
	amino-functionalized MCM-41					74.94	
	TBD-functionalized MCM-41	1	Methanol	1:4	318 K, 1.5 h	81.86	Deng et al. 2011
	Mg/Al oxides					95.2	
	Zinc oxide	1	Methanol	1:8	67°C, 100 min	98	Highina et al. 2011
	CaO		Methanol	1:12	70°C, 2.5 h	95	Hawash et al. 2011
	H_2SO_4	15	Methanol		60°C, 24 h	99.8	Shuit et al. 2009
Acid catalyzed	ZrO_2SO_4		Methanol			84%	Feng et al. 2008
	$K_4Zn_4[Fe(CN)_6]_3$		Methanol			90%	
	Lipase immobilized on celite (*Pseudomonas cepacia*)	10		1:4	40°C, 200 rpm	80	Shah et al. 2007
			Ethanol				
Enzymatic aprroaches	Lipase (*Chromobacterium visosum Candida rugosa, Porcine pancreas*)	10		1:4	40°C, 200 rpm, 8 h	62-71%	Shah et al. 2004b
	Lipase (*Enterobacter aerogenes*)	20		1:4	55°C after 48 h	94%	Kumari et al. 2009

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In addition, the activity of *Pseudomonas cepacia* lipase immobilized on celite was also investigated in the preparation of biodiesel from *Jatropha* oil (Shah et al. 2007). Ten percent of *Pseudomonas cepacia* lipase (by weight of oil) immobilized on celite showed the best yield of 98% of biodiesel produced in the presence of 4–5% (w/w) water in 8 h and at 50°C.

2.7.3. Supercritical alcohol transesterification

The transesterification of oils with non-catalytic supercritical alcohol has been developed to provide a new way of producing biodiesel (Koh et al. 2011). Supercritical alcohol transesterification is the method, in which solvent is subjected to temperature and pressure above its critical point. Under supercritical conditions no longer separate phase exist, only fluid phase is present and at a very high molar ratio of methanol to oil (42:1), the reaction is completed in a short time (Demirbas et al. 2005). The absence of catalyst in this process makes the glycerol recovery simple and biodiesel purification much easier and environmentally friendly (Encinar et al. 1999). However, the severe reaction conditions and high operational cost makes supercritical transesterification not a good option for industrial scale preparation. Therefore, for past few years has seen research focused on to decrease the severity of the reaction condition by using co-solvents, such as carbon dioxide, hexane, propane, calcium oxide or subcritical condition with small amount of catalyst to decrease the severity of operating conditions (Vyas et al. 2010). A few studies have carried out on supercritical alcohol transesterification of *Jatropha* oil. Hawash et al (2009) studied transesterification of *Jatropha* oil using supercritical methanol and in absence of a catalyst under different conditions of temperature (from 512 to 613 K), pressure (from 5.7 to 8.6 MPa) and molar ratio of alcohol to oil (from 10 to 43 mol of alcohol per mol of oil). They observed that 100% yield of *Jatropha* biodiesel obtained in 4 min using supercritical methanol (molar ratio of methanol to oil was 43:1) at a temperature of 593K (320°C) and under a pressure of

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8.4MPa. Similarly, Ilham et al. (2010) also reported a two-step supercritical dimethyl carbonate method for biodiesel production from *J. curcas* oil.

Chen et al. (2010) reported supercritical carbon dioxide extraction of *Jatropha* oil from seed, followed by subsequent subcritical hydrolysis and supercritical methylation of the extracted *J. curcas* oil and achieved 99% biodiesel yield at 11MP and 563K (290°C).

2.7.4 Microwave assisted transesterification

Microwave assisted transesterification is an alternative energy tool which can be utilized for the production of biodiesel. It offers a fast, easy route for biodiesel production by the application of radio frequency of microwave energy to enhance the speed of the reaction. The procedure offers easy separation, low oil to methanol ratio and reduced energy consumption and minimum by-products. However, the major drawbacks of using microwave irradiation for biodiesel synthesis are the scale-up of the process and its safety (Vyas et al. 2010). Several studies have been reported on microwave assisted transesterification of various bio oil (Nezihe et al. 2007; Nezihe et al. 2008; Perin et al. 2008). Shakinaz et al (2010) reported a new microwave technique for production of biodiesel from *Jatropha*. Their study showed that the application of radio frequency microwave energy accelerated the reaction rate from 150 min to 2 min and improved the separation process.

2.7.5 Ultrasound assisted transesterification

Ultrasonic technology is proven to be a useful technique in enhancing the reaction rates in many reacting systems. The ultrasound action in biodiesel production is primarily based on the emulsification of the immiscible liquid reactants by micro turbulence generated by radial motion of cavitation bubbles and the physical changes on the surface texture of the solid catalysts generating new active surface area (Veljokovic et al. 2012). The advantage of the process is faster reaction speed, with low molar ratio of methanol to oil and less energy consumption than for the conventional method (Ji et al.

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2006). Kumar et al (2010a) reported a new ultrasound-assisted transesterification method for *Jatropha* biodiesel production using Na supported on SiO_2 as a catalyst. A maximum yield of more than 98.53% of *Jatropha* biodiesel was obtained with 0.7 Hz ultrasonic irradiation pulse, 50% of ultrasonicwave amplitude within a reaction time of 15min. The methanol to oil ratio of 9:1 and 3% of solid Na/SiO_2 (by weight of oil) were found optimum conditions for this process.

2.8 Physicochemical property and quality of biodiesel

Biodiesels are best alternative for fossil fuel due to its physico-chemical properties, which are close to diesel fuel (Murugesan et al. 2009). Therefore, its use in diesel engine (neat or in blend) does not demand further modification (Tat and Gerpen 1999). Before using biodiesel in diesel engine, quality assessment is very important, otherwise it may lead to severe operational and environmental problem (Monteiro et al. 2008). The quality of biodiesel mainly depends on their physicochemical properties and contaminants. Usually ASTM standards are followed all over the world to check the quality of biodiesel. These properties determine the suitability a particular feedstock for the production of biodiesel. The properties such as oil content, cloud point, pour point, iodine value, oxidation stability, fatty acid profile, free fatty acid content, viscosity and acid number of biodiesel are dependent on the quality of the feedstock. Several researchers have investigated oil content, physicochemical properties, fatty acid composition and energy values of *Jatropha* oil and FAME all over the world (Table 2.2). Most of the studies conducted were incomplete or arbitrary in nature. However, no proper study has been conducted to study thermal and oxidative degradability of *Jatropha* oil and methyl esters. In addition to that, oil content and some of the physico-chemical properties (acid value, viscosity and fatty acid composition) of *Jatropha* collected from different location of the world showed variability. The variation in oil property mainly depended on origin and interaction of environment and genetics. But variation of these properties in methyl esters indicates variability in reaction conditions,

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temperature, catalyst, alcohol and the extent of reaction. As *Jatropha* is widely adapted in different climatic zone of the world, the study of all physicochemical parameter of oil is mandatory, as the process of biodiesel production, catalyst to be used, reaction condition depends solely on those properties. Moreover, the physicochemical properties of biodiesel produced should satisfy the specification of biodiesel standards for commercial use. The successful introduction and commercialization of biodiesel has been accompanied by the development of standards to ensure high product quality and user confidence (Knothe et al. 2005). Therefore, EN14214 (European committee for standardization) and ASTMD6751 (American society of testing materials) biodiesel standards are developed. The test methods and maximum allowable limits of physicochemical properties of biodiesel according to standard EN 14214-2008 and ASTM D 6751-09 and physicochemical properties of conventional diesel fuel is listed in Table 2.3.

Among the all physiochemical parameters studied for *Jatropha* oil collected from different geographical location of world, acid value is found to vary widely (Table 2.2). In literature, it is reported to vary from 0.92 to 29.8 mg KOH/g. Acid value is a measure of the free fatty acids (FFA) present in the oil. The variation in acid value may be due to wild origin, different maturation stage, seed storage (Oliveira et al. 2009) and edaphic condition of that region. The acid value of the oil determines the process of transesterification i.e. either one step or two step process. Depending on acid value one step base transesterification (Foidl et al. 1996; Rashid et al. 2010) as well as two step transesterification were done (Lu et al. 2009; Berchmans et al. 2009) for production of *Jatropha* biodiesel.

The kinematic viscosity of *Jatropha* oil also found to vary from 17.1 cst to 53.94 cst (Table 2.2). Viscosity of oil increases with increase in molecular weight and decreases with increase in unsaturation level and temperature (Nouredini et al. 1992). Highly viscous oil causes injector fouling and other engine operational problems.

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Different methods are reported like pyrolysis, micro-emulsification and transesterification to reduce viscosity of oil (Raheman et al. 2004). Transesterification process is most efficient process compared to others; it reduces the viscosity of oil, by forming esters molecules by removing glycerine from bulky triglycerides. Data obtained from literature showed that (Table 2.2), *Jatropha* oil showed viscosity in the range of 17.1- 53.94 cst, which further found to improve after transesterification (3.6-4.84 cst).

Density and specific gravities are also studied for *Jatropha* oil and biodiesel (Table 2.2). High-density biodiesel leads to incomplete combustion and particulate matter emissions (Rakhi et al. 2005). Density is found to be reduced after methanolysis of crude oil. Density of *Jatropha* oil and biodiesel studied were found in the range of 0.89-0.92 kg/m^3 and 0.82-0.88 kg/m^3 respectively (Table 2.2), which is within the tolerable limit of ASTM standards. Cloud and pour point of *Jatropha* oil was found around 5°C and -1°C respectively, which found to be reduced to -2°C and -5°C after transesterification (Table 2.2). Whereas, cloud and pour point of conventional diesel are around -16°C and -27° C respectively. This high cold property value of biodiesel is mainly due to presence of long chain saturated fatty acid (Stournas et al. 1995) in *Jatropha* oil. Various researchers suggested a number of possible ways such as transesterification, winterization, adding of additives to improve these cold flow properties (Dunn et al. 1996; Perez et al. 2010; Lee et al. 1996). Flash point of the *Jatropha* oils has been found in the range of 186°C to 240°C (Table 2.2). They were all above the than the minimum temperature of EN 14214 and ASTMD 6751 standards recommended range and therefore *Jatropha* biodiesel has an advantage of safe transportation and storage over diesel. Beside these, analysis of other important parameters like calorific value, Iodine value, moisture content and fatty acid compositions for *Jatropha* biodiesel have been shown to vary although within small data set. Literature survey showed that physicochemical properties of *Jatropha* oil varied with change in edaphic and climatic condition.

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Table 2.2 Physicochemical properties of the *Jatropha* biodiesel collected from different geographical region

Plant	Collection place	Acid value (mgKOH/g)		Viscosity (cst)		Density (g/cm³)		Cloud point (°C)		Pour point (°C)		Flash point (°C)		Reference
		Oil	FAME	Oil	FAME	Oil	FAME	Oil	FAME	Oil	FAME	Oil	FAME	
Jatropha curcas														
	Nicaragua (Africa)	0.92	0.24	52 (30°C)	4.84	0.92	0.88	-				240	191	Foidl et al. 1996
	Faisalabad (Pakistan)	-	0.40±0.03	-	4.80±0.17 (40°C)	-	0.88	-	10±0.1	-	6±0.2	-	188±3.0	Rashid et al. 2010
	Sichuan (South west china)	14	0.18-0.29	33.49-36.80 (40°C)	4.06-5.13	0.91	0.82	-	-	-	-	-	164-166	Lu et al. 2009
	Guizhou (China)	9.21± 0.03	0.04	-	4.312 (40°C)	-	0.88						147	Wang et al. 2010
	Nigeria (Africa)	3.5±0.1	-	17.1	-	-		-	-		-	-	-	Akintayo, 2004
	Bandung (Indonesia)	29.8	-	-	-	-	-	-	-	-	-	-	-	Berchmans et al. 2008
	Malaysia	4.46	-	42 (RT)	-	0.90	-	-	-	-	-	-	-	Akbar et al. 2009
	Brazil	8.45	-	30.68 (40°C)	4.02	0.92	0.88	-	-	-2	-5	-	117	Oliveira et al. 2009
	China	10.54	0.48	-	3.6 (40°C)	-	-	-	1	-	-	-	153	Qian et al. 2007
	Yunnan (South west China)	10.5	0.15	24.5 (40°C)	3.89	0.89	0.88			-2	-5	225	186	Deng et al. 2011
	Coimbatore (South India)	13.76	0.14	40.28	4.2	0.91	0.88	-				220	105	Bojan et al. 2011
	Nigeria	-	0.13	42.26 (30°C)	4.73 (40°C)	0.89	0. 86	-	-	-	-	216	184	Ojolo et al. 2011
	India	11	0.24	35.4 (40°C)	4.84	0.91	0.88	5	-	-6	-6	186	162	Singh et al. 2009
	Malaysia	4.46	-	47.50 (RT)	-	0.90	-	-	-	-	-	-	-	
	Indonesia	18.4	-	53.94 (RT)	-	0.90	-	-	-	-	-	-	-	Emil et al. 2010
	Thailand	3.38	-	39.20 (RT)	-	0.90	-	-	-	-	-	-	-	

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Table 2.3 Specification of biodiesel standards (Agarwal et al. 2007; Monteiro et al. 2008)

Properties	ASTM D 6751		EN 14214		Diesel
	Test method	Limits	Test method	Limits	
Density (kg/m3)	ASTM D 287	-	EN ISO 3675	0.86-0.90	0.83
Kinematic viscosity at 40°C (cst)	ASTM D445	1.9-6.0	EN ISO 3104	3.5-5	2.44±0.27
Flash point (°C)	ASTM D 93	130°C min	EN ISO 3679	>101	71±3
Fire point (°C)	ASTM D 92		EN ISO		103±3
Acid value (mg KOH/g)	ASTM D 664	0.5 max	EN 14104	0.5 max	9 -27
Iodine value	ASTM D 1951	>130	EN 14111	>120	-
Cloud point (°C)	ASTM D 2500	-	-	-	4.4
Pour pont (°C)	ASTM D 97	-	EN ISO 3016	-	-9.4 to -6.6
Calorific value (MJ/kg)	ASTM D 240	-	-	-	45.34

So, for development of commercial based biodiesel production in a region, a thorough study of all physiochemical parameters is essential for economic viability and success of the programme. Assam is a central state in North East India and rich in biodiversity. The species *J. curcas* is now well adapted in this unique bio geographic region. Due to the diverse climatic condition in this region, there is the possibility of existence of variation. Recently, government of Assam engaged Forest Development Agencies, self-help groups and other institutions in *Jatropha* activities for energy services as a way of finding a local solution for household fuel and income. However, these activities might hold the risk of unsustainable practices and loss of resources due to the lack of proper scientific knowledge and technology about the oil

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content and feasibility of this oil for production of fuel. No systematic study has been carried out on screening and identification of potential high oil yielding accessions and evaluation of its fuel properties of *J. curcas* from North East India for biodiesel production. Hence, there is an urgent need to investigate the oil content and physicochemical properties of *J. curcas* of this region. Based on this data, investors, policy maker and government can design realistic plan to get maximum production of biodiesel by reducing the shortcoming and environmental risk. The right cultivation and processing method can produce high quality bio-diesel which can compete with petro-diesel in terms of price and quality.

2.9 Life cycle assessment of the biodiesel production process from *Jatropha*

Lifecycle assessment (LCA) is a computational tool used to evaluate the environmental aspects and potential impacts associated with a product (ISO, 1997). Environmental processes are often very complex. This makes it difficult to model an LCA. Additionally LCA is often data intensive. Computers and adequate software tools are thus used to support the user in managing and editing these amounts of data. Therefore, LCA software is used to structure the modelled scenario, displaying the process chains and presenting and analysing the results. Several software tools like Simapro, GaBi, SIMBOX, Umberto, WISARD, ECO-it, CUMPAN, EcoLab, Eco Scan, EDIP PC-tool and GEMIS are available now to make the processing and calculation of LCAs simple.

A significant amount of the environmental problems of today are largely or partly caused by the utilization of fossil fuels. Release of carbon dioxide (CO_2) from fossil fuel is a major contributor in climate change and the global warming of the atmosphere (IPCC, 2007). Therefore, plant based biodiesel as an alternative fuel is gaining importance for energy independence and mitigation of global warming. However, the alternative fuel biodiesel adopted to reduce CO_2 fluxes in the atmosphere has come under intense scrutiny (Tilman et al. 2006, Field et al. 2007). Therefore, proper

investigation on environmental impact of biodiesel production will help to develop high energy efficient biofuel with positive effect on nutrient cycling in crop ecosystem with minimal land conversion (Field et al. 2007; Ragauskas et al. 2006; Fargione et al. 2008; Gurgel et al. 2007). Several studies has been conducted using LCA methodology to analyze the environmental impact of biodiesel production from renewable sources in different part of the world (Yu et al. 2009; Peiro et al. 2009; Requena et al. 2011; Nazir et al. 2010; Prueksakorn and Gheewala 2006; Pandey et al. 2011).

Spirinckx et al (1996) performed comparative life cycle assessment for rapeseed methyl ester and compared this with that of fossil diesel fuel, where they found specifically, the use of fertilisers has an important contribution on the emission of inorganic output and the production of landfill waste. They observed, only in fossil fuel consumption and global warming, biodiesel scores better than fossil diesel. A comprehensive life cycle assessment for biodiesel from tallow and used vegetable oil showed that regardless of the setting of system boundaries biodiesel from tallow and used vegetable oil performed better than fossil diesel and other biodiesel (Niederl et al. 2004). Investigation on the total energy requirement and global warming potential (GWP) of the production of rapeseed biodiesel in the UK showed transport involved in the various stages of manufacture had little effect on global warming potential (Stephenson et al. 2008). The environmental impact of soybean-based biodiesel production in Argentina showed as a soybean biodiesel exporter Argentina, would be competitive from the environmental point of view, only when some specific measures would be undertaken (Panichelli et al. 2009). These measures include avoiding deforestation, applying reduced tillage and crops successions, applying soybean inoculation methods, increasing yield, using low ecotoxicity pesticides and using biomass-based methanol or bioethanol in biodiesel production. Yu et al. (2009) summarized the study of the evaluations and comparisons of the three pilot projects, wheat-based, corn-based and cassava-based fuel ethanol (KFE) project from perspective

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of the economic viability and the long-term investment risk, energy efficiency and environmental emissions. The life cycle economy assessment showed that cassava based project was viable, while corn and wheat based projects are not without government's subsidies. Life cycle analysis of used cooking oil based biodiesel showed that the transesterification stage causes 68% of the total environmental impact (Peiro et al. 2009). Life cycle energy, environment and economic assessment for conventional diesel and soybean based biodiesel in China showed that compared to diesel, soybean biodiesel has similar source-to-tank total energy consumption but lower fossil energy consumption (Hu et al. 2008). Environmental and economic assessment of bioethanol production from sugarcane in Brazil showed that in terms of abiotic depletion, GHG emissions, ozone layer depletion and photochemical oxidation ethanol fuels were found better options than gasoline (Luo et al. 2009). But, gasoline was a better fuel where human toxicity, ecotoxicity, acidification and eutrophication were concerned. The results of life cycle costing (LCC) indicated that driving with ethanol fuels is more economical than gasoline.

Life cycle assessment of the biofuel production process from sunflower oil, rapeseed oil and soybean oil was found to show greater impact with respect to land use, fossil fuels, carcinogens, inorganic respiratory and climate change categories mainly due to the processes of seed production (Requena et al. 2011). Significant impact was also observed in drying and preparation processes of the seed as well as the crude soybean oil extracting process. Moreover, impact assessment showed that production of rapeseed and sunflower has a positive contribution to climate change.

The energy consumption of palm biodiesel production in Thailand using a life cycle approach showed that main contributors to the energy use were cultivation, oil production, transesterification and transportation (Pleanjai et al. 2007). Considering only fossil fuel in the production cycle, the energy analysis provides result in favour of palm biodiesel in Thailand. Yee et al (2009) conducted life cycle assessment (LCA) for

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palm biodiesel in order to investigate and validate the popular belief that palm biodiesel is a green and sustainable fuel. Results obtained showed that the utilization of palm biodiesel would generate higher energy yield than rapseed biodiesel, indicating generation of net positive energy and ensuring its sustainability. Papong et al. (2010) analyzed the energy performance and potential of the palm biodiesel production in Thailand. The results obtained showed that the highest fossil-based energy consumption was in the transesterification process, followed by the plantation, transportation, and palm biodiesel production. Angarita et al (2009) carried out study of energy balance in the Palm biodiesel life cycle taking into account practices in Brazil and Colombia. In the agricultural stage, for the two studied cases, the greatest contributions came from fertilizers. However, in the Brazilian case, this consumption is considerably less significant, as fertilizers were found to be used in relatively smaller quantities in adult palms' plantations. Comparing the energy balance values for biodiesel obtained from palm oil, rapeseed oil (Europe's), soybean oil (USA's) and castor oil (Brazil), it was observed that palm oil biodiesel is more energetically efficient than other biodiesels, and clearly more than fossil fuels (Angarita et al. 2009).

Development of non-edible oil-based biodiesel production can decrease the risk of food security. *J. curcas* is one of such non-edible vegetable oil, which has high oil content and its biodiesel shows high similarity to fossil fuel in fuel property. Through its suitability for wasteland cultivation, *J. curcas* provides two mechanisms for GHG abatement: substitution of fossil fuel and CO_2 sequestration through increasing carbon stocks above and below ground (Makkar et al. 2009). Globally, there are large areas of degraded croplands available in the developing world that is suitable for planting *J. curcas*. The establishment of this energy plant on such areas not only reduces GHG emissions but also creates employment opportunities for rural peoples. Therefore, *Jatropha* is gaining attraction, as a potential alternative source of renewable energy in recent years. However, proper investigation on environmental impact of biofuel crop is

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important determinates of the overall sustainability of biofuel as alternative fuel source. To be a viable alternative, a biofuel should provide a net energy gain, have environmental benefit, be economically competitive and be producible in large quantities without reducing food supplies (Hill et al. 2006). Several groups of researcher have performed life cycle analysis on different aspect of *Jatropha* biodiesel. Reinhardt et al (2007) conducted a screening LCA, examining the advantages and disadvantages of *Jatropha* biodiesel in India compared with conventional diesel. The study determined that *Jatropha* cultivation and processing made the greatest contribution to net GHG emissions, while the contribution of material transportation was minimal. Lam et al (2009) compared and validated the production of biodiesel from palm and *Jatropha* using the life cycle assessment (LCA) approach. Results obtained showed that to produce 1 tonne of *Jatropha* biodiesel, the land area requirement was 118% higher than to produce 1 tonne of palm biodiesel. The energy output-to-input ratio for palm biodiesel was found slightly higher (2.27) than *Jatropha* biodiesel (1.92). However, in contrary to the above result, Nazir et al (2010) showed superiority of *Jatropha* stock compared to palm biodiesel. According to them, biodiesel production from palm oil consumes much higher fossil-based energy than *Jatropha* oil. Prueksakorn et al (2006) investigated the energy consumption and greenhouse gas (GHG) emissions from *Jatropha* biodiesel production in Thailand. Main contributors to the energy use were found to be transesterification, irrigation, and fertilization process contributing approximately 40%, 23% and 22% respectively. Compared to the production and use of diesel, GHG emissions from JME were found to be about 77% lower. Sampattagul et al (2007) also developed the life cycle inventory database of *Jatropha* bio-diesel production in Thailand, which showed that major step of concern is cultivation process.

Whitaker et al (2009) evaluated the potential for *Jatropha*-based biodiesel in achieving sustainability and energy security goals. The life cycle impact of substituting petroleum diesel with biodiesel blends in Indian locomotives was also estimated. The

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result obtained suggested that substituting petroleum diesel with biodiesel blends yields reductions in both net GHG emissions and petroleum consumption.

Achten et al (2010) evaluated the life cycle energy balance, global warming potential, acidification, eutrophication and land use impact on ecosystem quality for a small scale, low-input *Jatropha* biodiesel system established on wasteland in rural India and compared with fossil fuel. The analysis of data showed that the production and use of *Jatropha* biodiesel triggers an 82% decrease in non-renewable energy requirement (Net Energy Ratio, NER = 1.85) and a 55% reduction in global warming potential (GWP) compared to the reference fossil-fuel based system. However, increase in acidification and eutrophication was found more from the *Jatropha* system relative to the reference case. For the land use impact, *Jatropha* was found to improve the structural ecosystem quality when planted on wasteland, but reduced the functional ecosystem quality. Fertilizer application (mainly N) was found as important contributor to most negative impact categories. Optimizing fertilization, agronomic practices and genetics are the major system improvement options.

Considerable variation was observed in the estimates of environmental impact with biodiesel production. This variation is mainly due to the variation in inventory compound and system boundary assumption. A study of environmental impact of biofuel crop cultivation and biodiesel production is very important, because it provides information that will enable effective measures to be undertaken so as to improve the GHGs and energy of the system. It will also enable out-growers, biofuel developers, policy makers and development practitioners to have better knowledge as to where and how to focus in the *Jatropha* production to bring a better environmental and economic achievements.

Our group has recently screened elite germplasm of North East India on the basis of oil content (37.63 to 46.6%) and physico-chemical properties (Mazumdar et al. 2012). Therefore, study on environmental performance of *Jatropha* biodiesel production

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from Assam could fully justify the overall sustainability of *Jatropha* biodiesel as energy source.

2.10 *In vitro* plant regeneration studies in *J. curcas*

J. curcas has received considerable attention as a potential source of non-edible vegetable oil which is eminently suitable for production of liquid biofuel (Azam et al. 2005; Tiwari et al. 2007). However, the major limitation in large-scale cultivation of this bioenergy crop is the low and inconstant seed yield due to heterozygous nature of plants and traditional propagation through stem cuttings is seasonal, prone to diseases and easy uprooting of established plants hamper the practical utility of this propagation method (Heller, 1996; Sujatha et al. 2005). Therefore, development of an efficient and reproducible micropropagation system may boost the mass propagation of this biofuel plant. Several protocols have been published in literature for plant regeneration of *J. curcas* using both direct and callus-mediated process, using shoot (Purkayastha et al. 2010; Rajore et al. 2005) and nodal meristems (Datta al. 2007; Kalimuthu et al. 2007; Sharma et al. 2010), cotyledonary leaf (Li et al. 2007; Mazumdar et al. 2010; Li et al. 2012; Khemkaladngoen et al. 2011) and leaf (Sujatha et al. 1996; Sujatha et al. 2005; Deore et al. 2008; Khurana-Kaul et al. 2010; Kumar et al. 2010b; Jha et al. 2007), hypocotyls (Sujatha et al. 1996; Kaewpoo et al. 2010; Sahoo et al. 2011; Li et al. 2012) and epicotyls segments (Qin et al. 2004; Kaewpoo et al. 2010) as well as mature (Shrivastava et al. 2009) and immature zygotic embryos (Varshney et al. 2010).

2.10.1 Direct organogenesis in *J. curcas*

Several research groups have reported direct organogenesis of *Jatropha* using various explants like leaf, petiole, node, shoot, shoot tips, hypocotyls, epicotyls, cotyledon, embryo and microshoot (Table 4). Mostly used plant growth regulator for initiation of direct organogenesis is TDZ, BAP, IBA and NAA alone or in various combinations. In most of the Euphorbiaceae members, presence of cytokinin alone gave optimal shoot proliferation such as 2-ip in *Euphorbia lathyrus* (Lee et al. 1982) and BA in *E. lathyrus*

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(Tideman and Hawker 1982; Ripley and Preece 1986). Sujatha and Mukta (1996) studied the morphogenesis and plant regeneration from hypocotyl, petiole and leaf explants. Higher regeneration was obtained from hypocotyl and petiole explants on MS (Murashige and Skoog 1962) media supplemented with BAP and IBA than on zeatin or kinetin supplemented media. Shoot bud induction was recorded highest on MS medium supplemented with 2.22 µM BA and 4.9 µM IBA, independent of the explants type. Combination of BAP and IBA found to effective for induction of shoot bud (Sujatha et al. 1993). Epicotyl explants of *J. curcas* showed adventitious shoot bud formation when cultured on MS medium containing BAP and IBA (Qin et al. 2004). Highest response for shoot bud induction was observed (38±2.1) in MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L IBA Sujatha et al (2005) has developed *in vitro* propagation system for non-toxic variety of *J. curcas* through axillary bud proliferation and direct adventitious shoot bud regeneration from leaf segments. Efficient adventitious shoot regeneration from leaf tissues was achieved when cultured on a medium containing 8.9–44.4 µM BA and 4.9 µM indole-3-butyric acid (IBA) followed by transfer to medium supplemented with 8.9 µM BA and 2.5 µM IBA. Rajore and Batra (2005) cultured shoot tip explants MS media supplemented with 8.87 µM BAP and 2.85µM IBA along with additive adenine sulphate, glutamine and activated charcoal. The regenerated shoots are rooted on half strength MS media supplemented with 14.7 µM IBA. Combination of 2.22 µM BAP and 0.049 µM IBA was the most effective for multiple shoot bud formation from axillary bud explants of *J.curcas* (Thepsamran et al. 2007). After multiple shoot formation, individual shoots are cultured on MS medium for rooting. Shoots produced roots after 5 weeks with 50% rooting efficiency. Datta et al (2007) reported axillary shoot bud proliferation from nodal explants. Best response (6.2±0.56) shoot per nodal explants) was obtained when explants were cultured on MS media supplemented with 22.2µM BAP and 55.6µM adenine sulphate. Whereas, Shrivastava et al (2008) reported a efficient regeneration of axillary node, when cultured MS media containing BAP (3.0

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mg/L) and IBA (1.0 mg/L) supplemented with growth additive (adenine sulphate, glutamine and L-arginine). TDZ was found very effective in inducing high-shoot buds in tissue culture of woody plants (Huetteman and Preece 1993; Meng et al. 2004) than only BAP. Deore and Johnson (2008) developed an adventitious shoot bud regeneration system using leaf explants. The leaf explants were cultured on MS medium supplemented with 2.27 µM thidiazuron (TDZ), 2.22 µM BAP and 0.49 µM IBA followed by transfer to a MS medium supplemented with 4.44 µM BA, 2.33 µM kinetin (Kn), 1.43 µM indole-3-acetic acid (IAA) and 0.72 µM gibberellic acid (GA_3) showed best response. The results obtained from the experiment, support the influential role of TDZ on high-frequency shoot-bud induction from *J. curcas* leaf discs. Singh et al (2009) reported direct regeneration from stem explants of *J. curcas*. Shoot bud induction (10–15 buds per explants) was achieved on MS medium supplemented with 1.0 mg/L BAP, in combination with 1.0 mg/L 6-furfurylamino purine. Our lab also developed a regeneration system for *J. curcas* using shoot apices, which was found suitable for gene transfer by particle bombardment (Purakayastha et al. 2010). Maximum shoot was obtained (6.2) in MS media supplemented with 2.5µM BAP. Khurana-Kaul et al (2010) reported direct shoot organogenesis from leaf explants of *J curcas* using TDZ. Adventitious shoot buds developed directly on the surface of the explants without formation of intervening callus, when the leaf explants were cultured on MS medium supplemented with 0.90 µM TDZ and 0.98 µM IBA. In addition to that, effect of copper sulphate on differentiation of shoot buds from leaf segments was also investigated. Significant improvement in shoot bud induction was acheived when the concentration of $CuSO_4$ was increased to 10 times than normal MS level. Sahoo et al (2011) obtained maximum 12.1 shoot per explants, when hypocotyl explants of *J. curcas* were cultured on MS medium supplemented with 7.05 µM kn and 1.425 µM IAA. Rooting of shoots was best achieved on half-strength MS medium supplemented with 9.8 µM IBA. Kumar et al (2011) reported a simple and efficient protocol for plant regeneration using

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cotyledonary petiole explants of non-toxic variety of *J. curcas*. The concentration of TDZ, orientation, and source of explants on shoot buds induction was evaluated.

Table 2.4 Currents status on regeneration studies of *J. curcas*

Explant used	Mode of regeneration	References
Hypocotyl, petiole and leaf	Regeneration via adventitious shoots, rooted *in vitro*	Sujatha and Mukta 1996
Leaf	Somatic embryogenesis	Sardana et al. 2000
Axillary node and leaf	direct adventitious shoot regeneration	Sujatha et al. 2005
Leaf	direct adventitious shoots	Deore and Johnson 2008
Leaf	direct organogenesis	Khurana-Kaul et al. 2010
Leaf	direct organogenesis	Kumar et al. 2010c
Leaf	Somatic embryogenesis	Jha et al. 2007
Leaf and hypocotyl explants	Callus and suspension culture	Soomro and Memon 2007
Petiole	direct organogenesis	Kumar and Reddy 2010d
Petiole	direct organogenesis	Kumar et al. 2010e
Petiole, Hypocotyl	indirect organogenesis	Li et al. 2012
Nodal	Direct organogenesis	Datta et al. 2007
Nodal segment	Somatic embryogenesis	Kalimuthu et al. 2007
Nodal segment	Direct organogenesis	Sharma et al. 2010
Axillary node	Direct shoot regeneration	Shrivastava and Banerjee 2008
Axillary bud	Multiple shoot induction	Thepsammran et al. 2007
Stem	direct organogenesis	Singh et al. 2010
Leaf	Direct and indirect organogenesis	Misra et al. 2010
Shoot tips	Direct organogenesis	Rajore and Batra 2005
Shoot tips	Direct organogenesis	Purkayastha et al. 2010
Hypocotyl and epicotyl	Direct and indirect organogenesis	Kaewpoo and Te-chato 2010
Hypocotyl	Direct organogenesis	Sahoo et al. 2011
Epicotyl	Direct organogenesis	Qin et al. 2004
Cotyledon (14 days)	indirect organogenesis	Li et al. 2007
Embryogenic cotyledons	indirect organogenesis	Mazumdar et al. 2010
Cotyledon	Direct shoot regeneration	Kumar et al. 2010b
Juvenile cotyledon	Direct organogenesis	Khemkaladngoen et al. 2011
Embryo	Direct organogenesis	Shrivastava et al. 2009
Immature embryo	In direct organogenesis	Varshney et al. 2010
Microshoot	Direct organogenesis	Shah et al. 2010

The percent of induction of shoot buds and the number of induced shoot buds per explant was found directly proportional to the concentration of TDZ. However, higher concentration of TDZ further inhibits the proliferation and elongation of shoot buds. The best induction of shoot buds (59.11%), and highest number of shoot buds (5.01) per explants were obtained, when explants were cultured on MS medium supplemented with 2.27 µM TDZ and cultured in the horizontal position. *In vitro* explants responded efficiently as compared to in vivo explants. However, Khemkladngoen et al (2011)

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showed that, combination of BAP (3mg/L) and IBA (0.1mg/L) alone are sufficient for adventitious shoot regeneration from juvenile cotyledons. Misra et al (2010), reported direct shoot organogenesis from exvitro leaf explants, when cultured on MS medium supplemented with 0.5mg/L BAP and 0.1 mg/L IBA.

Direct organogenesis study in *Jatropha* showed, BAP plays very important role in shoot induction (Purakaystha et al. 2010). Combination of BAP and IBA also found very effective in direct shoot regeneration in various explants (Sujatha and mukta 1996; Qin et al. 2004; Khemkladngoen et al. 2011). In contrary to the above result, Deore and Johnson, 2008 showed addition of TDZ with combination of BAP and IBA had higher impact on initiation shoot bud. Whereas, Kumar et al (2011) showed only TDZ is more effective in shoot bud initiation compared to BAP.

2.10.2 Indirect organogenesis in *J. curcas*

A few reports are available in literature on indirect organogenesis of *J. curcas* (Table 4). Sujatha and Mukta (1996) studied the indirect organogenesis of *J. curcas* using mature leaves. Highest no of shoot per explants (10.7) obtained on MS medium supplemeted with 2.22 µM BA and 2.46 µM IBA. Soomro et al (2007) has reported induction of callus and suspension culture using hypocotyl explants. Li et al (2007) reported a regeneration system via callus using 14 days old cotyledon for *Agrobacterium* mediated transformation. However, the system found to be inefficient as age of the explants is not studied. The age of the explant is a critical aspect in regeneration and as well as in transformation. Therefore, we have evaluated the effect of the age and orientation of the cotyledonary leaf explants on plant regeneration and *Agrobacterium*-mediated transformation (Mazumdar et al. 2010). The result obtained showed that, cotyledonary leaf segment explants prepared from seeds, cultured with their abaxial surface placed in the medium showed significantly better regeneration response as compared to older explants and the explants cultured with their adaxial surface in culture medium. Khurana-kaul et al (2010) observed that leaf explants produced adventitious shoot buds

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without formation of any callus, when cultured on combination of TDZ and IBA, while shoot bud formation was accompanied with callus formation on medium supplemented with 13.3 µM BAP and 2.46 µM IBA. Varshney et al (2010) investigated the morphogenic potential of immature embryo of *J. curcas*. Immature embryo explants of four developmental stages were cultured on medium supplemented with combinations of auxins and cytokinins. Embryo size was found critical for the establishment of callus. Immature embryos (1.1–1.5 cm) obtained from the fruits 6 weeks after pollination showed a good response of morphogenic callus induction (85.7%) and subsequent plant regeneration (70%) with the maximum 4.7 shoot per explants on MS medium supplemented with 0.5 mg/L IBA and 1.0 mg/L BAP. In addition to that, plant regeneration (90%) found to be improved more after addition of adjuvant like casein hydrolysate (100 mg/L), L-glutamine (200 mg/L) and $CuSO_4$ (8.0 mg/L) on medium. Recently Li et al (2012) studied plant regeneration through callus induction using epicotyl, hypocotyl, petiole and cotyledon of 8-day-old seedlings of *J. curcas*. Results obtained demonstrated that the combination of 1 mg/L NAA and 0.1 mg/L Kn was the best medium for callus induction and growth. Induced calli transferred to regeneration medium containing 1 mg/L TDZ and 1 mg/L Kn combined with 0.1 mg/L IBA showed best respond in shoot development.

2.10.3 Somatic embryogenesis in *J. curcas*

Only three reports are available on literature on plant regeneration of *J. curcas* through somatic embryogenesis (Table 2.4). Sardana et al (2000) first time reported, plant regeneration through somatic embryogenesis in *Jatropha*. Embryogenic callus and globular embryos were formed from leaf explants cultured on Murashige & Skoog's (MS)-Gamborg's medium (containing MS basal salts and Gamborg's vitamins), supplemented with 3 mg/L BAP and IAA. Next stage is followed by induction of plantlets from somatic embryo, after transferred to MS media supplemeted with 3 mg/L gibberellic acid (GA_3) and 1 mg/L IAA. Whereas, Jha et al. (2007) reported induction of

embryogenic callus and globular somatic embryo from leaf explants in MS medium supplemented with only 9.3 µM Kn and MS medium supplemeted with 2.3 µM Kn and 1.0 µM IBA respectively. Kalimuthu et al. (2007) reoprted the direct somatic embryogenesis from green cotyledon explants of *J. curcas* on MS medium fortified with 2 mg/L of BAP. The results of histological studies showed that several layers of meristematic cells participated in the formation of somatic embryos. In addition to this, the result obtained from this study also showed that, the synergistic combination of reduced Kn, IBA and adenine sulphate not only promoted the growth of shoot and root but also secondary embryo formation.

2.11 Current status of transgenic research in *Jatropha*

Development of genetic transformation system in *Jatropha* has become very necessary for trait improvement of this biofuel crop as well as for understanding functional genomics of plant. Several researchers in recent year have been developing different transformation methods for *Jatropha* to achieve this goal (Table 2.5).

2.11.1 Genetic transformation of *J. curcas* by microprojectile bombardment

Our lab reported for the first time, gene transfer by particle bombardment in *J. curcas* (Purkayastha et al. 2010). We established a genetic transformation system using shoot apices as explants. Explant was bombarded with particles coated with plasmid pBI426 with a GUS-NPT II fusion protein under the control of a double 35S cauliflower mosaic virus (CaMV) promoter. The result obtained from the experiment showed that, β-glucuronidase (GUS) activity in *J. curcas* shoot apices was significantly affected by the gold particle size, bombardment pressure and target distance, macro carrier travel distance, number of bombardments, type and duration of osmotic pre-treatment. Preculture of shoot apices on 0.2 M mannitol (osmotic treatment) for 4 h prior to bombardment was found to improved transient GUS expression The highest frequency of transient gus expression was observed when explants were bombarded at 1100 psi with a target distance 9 cm. After that, Joshi et al (2011) reported genetic

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transformation of *Jatropha* by microprojectile bombardment using embryo axes. They also found that, microcarrier size, helium pressure and target distance had significant influence on transformation efficiency. Among different variables evaluated, microcarrier size 1µm, He pressure 1100 and 1350 psi with a target distance of 9 and 12 cm respectively were found to show high gus expression and survival of putative transformants. Although frequency of transient gus expression increases with increase in helium pressure and microcarrier size but there was a reduction in the frequency of regeneration due to tissue damage. The results obtained from the experiment showed the possibility of stable transformation of *Jatropha* through direct gene transfer method with 44.7% transformation efficiency.

2.11.2 *Agrobacterium* mediated genetic transformation in *J. curcas*

Agrobacterium tumifaciens mediated transformation is most preferred method of gene delivery, due to its simplicity, cost effectiveness, defined integration of transgene in to the host genome (Koncz et al. 1989; Hamilton et al. 1996; Ingelbrecht et al. 1991). First report on *Agrobacterium* mediated genetic transformation of *Jatropha* from Chinese accessions was done by Li et al (2007). Explant used was cotyledonary disc. Transformation was carried out with *Agrobacterium* cells harbouring p3301-BI121-SaDREB1 plasmid, which contain SaDREBI gene with bar gene for selection on phosphinothricin and β-glucuronidase (gus) as reporter (cloned from PBI121) gene and pCAMBIA1301carring both gus and the select-able marker gene for hygromycin phosphotransferase (*hpt*). The result obtained from the experiments revealed significance influence of *Agrobacterium* strain (LBA 4404 and EHA 105), selection agent (phosphinothricin, hygromycin and kanamycin) and bacterial incubation time (5–30 min) on transformation efficiency of *J. curcas*. Maximum transformation efficiencies were achieved with LBA 4404 strain (OD600 of 0.4–0.5) with bacterial incubation period of 10 min at 28°C, 3 days of cocultivation at 25°C in a co-cultivation medium containing 20mg/L acetosyringone, followed by selection on phosphinothricin and

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hygromycin. Presence of the gene in primary transformants was confirmed through PCR and Southern analysis. The overall transformation efficiency was achieved around 13%. Trivedi et al (2009) also reported an *Agrobacterium*-mediated genetic transformation system in *J. curcas* using cotyledonary explants, however the study was only confined to callus. The major focus of the work was to study the Agro-infection of leaf explants from cotyledons and integration of transgene in the callus. Transformation was carried out with *Agrobacterium* LBA4404 strain harbouring pCAMBIA1301*S-DREB2A* containing *hpt* gene, as selectable marker and *gus* as reporter gene. Sensitivity of cotyledons and callus to antibiotics was tested and 5 mg/L of hygromycine were found optimum for selection of transgenic. Effect of different concentration of Acetosyringone (50, 100, 200 and 400 μM), dipping time of cotyledonary leaf explants in *Agrobacterium* suspension was also investigated. They observed in all concentration of Acetosyringone and dipping time 6h, 100% infection was visible in explants. Whereas, in case of 12h dipping, it was found lethal for all explants. Li et al (2007) established an *Agrobacterium* transformation system in *Jatropha* using 14 days cotyledonary explants. Transformation was carried out with *Agrobacterium* LBA4404 strain harbouring p3301-BI121-*SaDREB1* containing *bar* gene, as selectable marker and *gus* as reporter gene. However, the above transformation systems optimized found to inefficient or difficult to reproduce as age of the explants is not studied. The age of the explant is a crucial aspect in transformation experiment and therefore, appropriate biological condition of the explant is vital for optimal infection and T-DNA transfer by *Agrobacterium* tumefaciens (Purkayastha et al. 2010). Therefore, we have evaluated the effect of the age of the cotyledonary leaf explants on *Agrobacterium*-mediated transformation (Mazumdar et al. 2010). Transformation was carried out with EHA105 *Agrobacterium* strain harbouring pCAMBIA2301 containing neomycin phosphotransferase (*nptII*) gene, conferring kanamycin resistance and *gus* reporter gene. Previous transformation studies in *Jatropha* was attempted with only with bar and hygromycin antibiotics. Maximum gus expression was observed in

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embryogenic cotyledons infected with *Agrobacterium* culture corresponding to OD600 = 0.8 for 30min, followed by co-cultivation for 3 days at 25 °C in a co-cultivation medium containing 100 µM acetosyringone, pH 5.7. In contrary to the Trivedi et al (2009) acetosyringone concentration more than 100µM was found to inhibit callus induction. Cotyledonary leaf segment, prepared from freshly germinated seeds were found to be most amenable to *Agrobacterium*-mediated transformation as compared to older explants. Later on, Zong et al (2010) reported a transformation method, using young leaf as explants. Based on that procedure the lateral shoot inducing factor (LIF) from Petunia has been introduced in to *J. curcas*. Transformation was carried out with LBA4404 strain harbouring PBI121-*LIF* recombinant plasmid, containing gus as reporter gene and neomycin phosphotransferase (*nptII*), as selectable marker. LIF is a zinc finger protein isolated from Petunia and overexpression of this protein in petunia, tobacco and Arabidopsis resulted dramatic increased in lateral shoot and reduced height (Nakagawal et al. 2005). Contrary to the previous report, they observed LBA4404 and EHA105 had the same virulence on young leaf of *Jatropha*. The sensitivity of *J .curcas* tissues to cefotaxime (300mg/L) and the selection agents kanamycin (40mg/L) was optimized. Maximum transformation efficiencies were achieved with bacterial incubation period of 10 min, 5 days of cocultivation followed by selection on kanamycin. Presence of the gene in primary transformants was confirmed through PCR and Southern analysis. The overall transformation efficiency was achieved around 23.91±5.78%. Pan et al (2010) reported an *Agrobacterium*- mediated transformation protocol using cotyledonary explants. *Agrobacterium* strain LBA4404 harboring the binary vector pCAMBIA2301, which contains nptII and gus gene was used for transformation experiments. Kanamycin was observed to inhibit callus induction. So, kanamycin was not included in callus induction medium. 20 mg/L kanamycin and 100 mg/L cefotaximum was found optimum for selection of transgenic shoots from callus. PCR and Southern analysis confirmed the presence of transgene. Transformation parameter like, incubation period of 20 min with

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agitation at 200 rpm (at room temperature) followed by cocultivation of 3 days at 26±2°C were found optimum for successful transformation.

Kumar et al (2010f) studied the key factors affecting *Agrobacterium*-mediated transformation such as preculture of explants, wounding of leaf explants, *Agrobacterium* growth phase (OD), infection duration, co-cultivation period, co-cultivation medium pH, and acetosyringone with the aim of improving transformation in *J. curcas*. *Agrobacterium* strain LBA 4404 harbouring the binary vector pCAMBIA 1304 having sense-dehydration responsive element binding (*S-DREB2A*), gus and hpt genes were used for transformation studies. The highest transformation efficiency was achieved using 4-day precultured, non-wounded leaf explants infected with *Agrobacterium* culture corresponding to OD600=0.6 for 20min followed by co-cultivation for 4 days. Co-cultivation medium containing 100 µM acetosyringone, pH 5.7 was observed optimum in their experiments, which is in agreement to with our result (Mazumdar et al. 2010). GUS histochemical analysis of the transgenic tissues confirmed the transformation event and PCR and DNA gel blot hybridization confirmed the presence of transgene. A transformation efficiency of 29% was achieved for leaf explants using this protocol.

Khemkladngoen et al (2011) investigated 1-min sonication treatment of cotyledonary explants followed by 9 min shaking in *Agrobacterium* suspension is an effective protocol for obtaining the transformation of *J. curcas* with a high stable transformation efficiency (approx. 53%). LBA4404 containing the binary vector pBI121containing *nptII* and *gus* gene was used for transformation treatments. Stable transformation and gene integration was confirmed by PCR and southern hybridization. Wounding of the tissues by sonication and sand-vortexing in addition to shaking were found to facilitate efficient *Agrobacterium* infection which is contrary to the report of Kumar et al. (2010f), where they have showed experimentally, wounding treatments by glass beads and hand pricking with needle reduces transformation efficiency. However, stable transformation efficiency (approx. 53%) was found much higher compared to the

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report of Kumar et al 2009 (29%) and Li et al 2007 (13%). Misra et al (2012) reported an *Agrobacterium* mediated transformation protocol for *J. curcas* using invitro Leaf and hypocotyl segments.

Transformation of *Jatropha* studies using both of these explants has not been reported earlier, as all previous studies in *Jatropha* were done using cotyledonary and leaf explants. *Agrobacterium* strain EHA101, harboring the binary vector pIG121Hm containing *nptII* as selectable marker and *gus* as repoter gene were used for all transformation studies. The super virulent strain EHA101 was found very effective with distinct blue visible spots in both the explants, whereas, LBA4404 did not show any GUS expression, which is contrary to the previous published report (Li et al. 2007). Both types of explants showed similar transient GUS expression after 3, 4 or 5 days of cocultivation. However, 5 days led to overgrowth of *A. tumefaciens*, killing the explants, whereas, after 3rd day 80% of the explants survived after cocultivation. Screening of transgenic was done by PCR of *gus* reporter gene and formation of transcript was confirmed by RT-PCR of *gus* gene.

Stable transformation efficiency was observed 5% in case of leaf explants and whereas 4% in hypocotyl explants, which is found very low compared to previous published reports.

Most of the reports are based on optimization of *Agrobacterium* mediated transformation in *Jatropha*. To date, only two reports are available on generation of stable transgenic plants using candidate genes (Qu et al. 2012; Tsuchimoto et al. 2012). Qu et al (2012) developed marker free transgenic *Jatropha* plant with increased levels of seed oleic acid. *JcFAD2-1*, a delta 12 fatty acid desaturase gene in *Jatropha* was down regulated in a seed-specific manner by RNA interference technology.

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Table 2.5 Transformation studies in *J. curcas*

Explants Used	Mode of regeneration	DNA Delivery method	Strain/Vector/construct	Selection	Frequency of transformation (%)	References
14 days old cotyledon	De novo	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 3301-BI121-*SADREB1*	*hptII*	13 %	Li et al. 2007
cotyledon	De novo	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 1301S-DREB2A	*hptII*	-	Trivedi et al. 2009
Young leaf	De novo	*Agrobacterium* mediated transformation	LBA4404 PBI121-*LIF*	*nptII*	23.91±5.78	Zong et al. 2010
Cotyledon	De novo	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 2301	*nptII*	-	Pan et al. 2010
Juvenile cotyledons	Direct organogenesis	*Agrobacterium* mediated transformation	LBA4404 PBI121	*nptII*	53 %	Khemkladngoen et al. 2011
Leaf	Direct organogenesis	*Agrobacterium* mediated transformation	LBA4404 PCAMBIA 1304-*S-DREB2A*	*hptII*	29 %	Kumar et al. 2010f
Shoot apices	Direct organogenesis	Biolistic gun	pBI426	*nptII*	-	Purkayastha et al. 2010
Embryo axes	Direct organogenesis	Biolistic gun	PCAMBIA 1301	*hptII*	44.7 %	Joshi et al. 2011
Cotyledon	De novo	*Agrobacterium* mediated transformation	PCAMBIA 1300 JcFAD2-1 RNAi	*hptII*	-	Qu et al. 2012

The resulting *JcFAD2-1* RNA interference transgenic plants showed a dramatic increase of oleic acid (> 78%) and a corresponding reduction in polyunsaturated fatty acids (< 3%) in its seed oil, whereas, control *Jatropha* had around 37% oleic acid and 41% polyunsaturated fatty acids.Thereafter, Tsuchimoto et al (2012) reported development of transgenic Jatropha expressing three drought tolerant genes, the PPAT gene, which encodes an enzyme that catalyzes the CoA biosynthetic pathway; the second overexpresses the NF-YB gene,which encodes a subunit of the NF-Y transcription factor; and the last overexpresses the GSMT and DMT genes, which encode enzymes that catalyze production of glycine betaine. They screened transgenic plant on the basis of PCR. Quantitative analysis of glycin betain accumulation was showed introduction of GSMT and DMT gene significantly enhances glycine betain synthesis in Jatropha. However, intergration, expression and physiological analysis for drought resistance data for transgenic plant have not been shown.

2.11.3 Opportunities of genetic improvement of *Jatropha* for insect pest resistance

Jatropha crop is projected as less prone pest damage due to the lack of systematic survey. There was a popular belief that toxicity and insecticidal properties of *J. curcas* are sufficient repellent for insects that cause economic damage. However in reality, *J. curcas* has found susceptible to several groups of insect and pest in large scale plantation (Shanker and Dhyani 2006). In its native range, *Jatropha* has been found susceptible to more than 40 species of insects including the shieldbacked bug (*Pachycoris klugii*) and the leaf-footed bug *Leptoglossus zonatus* (Grimm & Fuhrer 1998; Grimm & Somarriba 1999). More than a dozen pests occurring in *J. curcas* plantations in Udumalpettai, Erode and Mettupalayam areas of Tamil Nadu was reported by Manoharan et al (2006). Among the insect and pest observed on *J. curcas*, *S. litura* is found as an extremely serious pest, the larvae of which can defoliate many economically important crops. About 120 kinds of plant are used as host plant by this pest, such as tobacco, corn, paddy, tomato, chili and legumes including soy bean, *J.*

curcas and taro. Meshram et al (1994), first time reported *S. litura* as a pest of *J. curcas* and was found to attack 60 to 70 percent *Jatropha* crop in a plantation in Madhya Pradesh. The larva of this polyphagous insect was also observed to feed on *J. curcas* leaves over the northern two-thirds of Australia (Herbison-Evans et al. 2006). Distribution of this *S. litura* is very wide especially in Asia, Pacific and Australia. In controlled experiments on soyabeans in India, crops protected from *S. litura* and other pests yielded over 42% more than crops which were not protected (Srivastava et al. 1972). On tobacco cultivation, in India, it was estimated that two, four and eight larvae per plant reduced yield by 23-24%, 44.2% and 50.4%, respectively (Patel et al. 1971). On *Colocasia esculenta*, an average of 4.8 4th-instar larvae per plant reduced yield by 10%, while 2.3 and 1.5 larvae reduced yield of Aubergines and Capsicum in glasshouses by 10% (Nakasuji et al. 1976). Sustainable and ecologically acceptable means of control of pests and diseases are of paramount importance, if large scale *Jatropha* plantations have to develop successfully. Otherwise, with taking up elite *Jatropha* varities as regular monocrop in continuous stretches not only in marginal lands but also in arable lands, outbreak of pests can happen anytime.

Currently, insecticide sprays are the only viable control technology available, but this approach has been compromised with the emergence of resistant biotypes due to indiscriminate use of multiple classes of insecticides (Ekesi, 1999). Therefore, development of *J. curcas* cultivars with insect resistance can form the backbone for integrated management of these insect and pests. Conventional breeding can been exploited to increase the genetic diversity of the crop as well as to screen *Jatropha* genotypes to identify resistance varieties. However, this conventional breeding approach, faces many challenges, not least being the length of time involved for resistance gene discovery and the possibility that resistance genes may not be present in the *Jatropha* germplasm.

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It is known that insect pests, especially lepidopterans, can be controlled by *Bacillus thuringiensis* (Bt) – an ubiquitous, soil-dwelling, spore-forming bacterium – when applied topically on crops as spore formulations. Incidentally, Bt sprays are often washed away by rain, degrade under solar ultra violet radiation and are not optimally targeted against certain insect pests that live within plant tissues. Therefore, the application of genetic engineering tool holds great promise in overcoming the constraints in *Jatropha* production by expressing candidate genes for insect resistance. Deployment of crystal toxin genes from *B. thuringiensis* in *Jatropha* can build protection against target lepidopteran insects through the conceptual framework of genetic transformation. Transgenic oil seed crops including canola (Stewart et al. 1996), oil palm (Lee et al. 2006) and castor (Kumar et al. 2011) expressing cry gene demonstrated effective resistance against target insects. Pigeon pea (Sharma et al. 2006) expressing cry gene was shown to have an effective resistance against *Spodoptera litura*. Activated Cry1Ac toxin expressed in Bt cotton (Monsanto line NuCOTN-33B) caused an increase in mortality, a delay in development, and a decrease in the body mass of first instar *S. litura* (Zhang et al. 2006) suggesting that generation of transgenic *Jatropha* expressing *cry1Ac* may enable to confer resistance against target insect.

2.11.4 Mode of action of Bt cry genes for developing resistance against target insect

The source of cry genes is *B. thuringiensis* (Bt), a Gram-positive facultative-aerobic soil bacterium. It synthesizes crystalline insecticidal proteins known as ó-endotoxins or Cry proteins during sporulation (Sanchis et al. 2008). Bt endotoxins have been used as biological control reagents since the 1920's, but use of specific Bt toxins has increased dramatically since 1996, with the introduction of these candidate Bt genes in crops through conceptual framework of genetic engineering. These transgenic plants were significantly toxic to the target insects while there was no direct effect on non-target species (Qaim and

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Zilberman 2003). The mode of action of Cry toxins has mostly been studied in lepidopteran insects (Bravo et al. 2011).

Cry toxins are classified by their primary amino acid sequence and more than 500 different cry gene sequences have been classified into 67 groups (Cry1-Cry67) (Crickmore et al. 2005). These cry gene sequences have been divided in to at least four phylogentically non-related protein families that may have different modes of action: the family of three domain Cry toxins (3D), the family of mosquitocidal Cry toxins (Mtx), the family of the binary-like (Bin) and the Cyt family of toxins (Bravo et al. 2005; Bravo et al. 2011). The three dimensional structure of seven different 3D-Cry toxins have been solved, Cry1Aa, Cry2Aa, Cry3Aa, Cry3Ba, Cry4Aa, Cry4Ba and Cry8Ea (Li et al. 1991; Grochulski et al. 1995; Morse et al. 2001; Galitsky et al. 2001; Boonserm et al. 2005; Guo et al. 2009). The three-dimensional structures of Cry toxins showed that they are made up of three domains. Domain I, made up of seven –helices is in membrane insertion, toxin oligomerization and pore formation (Pigott et al. 2007, Bravo et al. 2011). Domain II consists of three antiparallel β-sheets packed around a hydrophobic core with exposed loop regions that are involved in receptor recognition. Structurally, domain II is the most variable of the toxin domains (Boonserm et al. 2005). Due to this variability, domain II is believed to be an important determinant of toxin specificity. Domain III is a β-sandwich of two anti-parallel β -sheets. Domain III shows less structural variability than domain II, and the main differences are found in the lengths, orientations, and sequences of the loops. Both domain II and III are implicated in insect specificity by mediating specific interactions with different insect gut proteins (Bravo et al. 2004). The three dimensional structure is conserved among members of the 3D-Cry family suggesting proteins from this family may share a similar mechanism of action even though they show very low amino acid sequence similarity (Bravo et al. 2011). Full length Cry toxins are inactive until cleaved to generate their active

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form in the insect midgut (Lemaux, 2009) (Fig. 2.6). The cry genes code for proteins with arrange of molecular masses from 50 to 140 kDa. They are cleaved in vivo by the digestive proteases of the insect host to generate mature toxins of about 60-70 kDa (the amino-terminal part of the pro-toxin) (Babendreier et al. 2005). The primary action of Cry toxins is to lyse midgut epithelial cells in their target insects by forming lytic pores in the apical microvilli membrane (Schnepf et al. 1998; de Maagd et al. 2001; Bravo et al. 1992). The activated toxin binds to specific receptors on the brush border membrane of the midgut columnar cells (Schnepf et al. 1998; Bravo et al. 1992). It was proposed that the toxin receptor interaction triggers toxin oligomerization resulting in the formation of a soluble pre-pore (Gomez et al. 2002). The pre-pore inserts into the membrane, forming lytic pores (Bravo et al. 2004). Cell lysis and the disruption of the midgut epithelium provide a rich medium suitable for spore germination leading to severe septicaemia and insect death (Schnepf et al. 1998; de Maagd et al. 2001).

Cry toxin binding to insect midgut epithelial receptors is an important determinant of specificity. The toxin receptor interaction is a complex process, involving multiple interactions with different receptor and carbohydrate molecules (Lopez et al. 2006). It has been proposed that Cry1A toxins sequentially interact with a cadherin receptor, leading to the formation of a pre-pore oligomer structure, and that the oligomeric structure binds to glycosylphosphatidyl-inositol-anchored minopeptidase-N (APN) receptor. The Cry1Ac toxin specifically recognizes the N-acetylgalactosamine (GalNAc) carbohydrate present in the APN receptor (Lopez et al. 2006). Several research groups working in the domain of plant transgenes is decided to make use of the insecticidal potential of Bt to generate genetically modified plants expressing δ-endotoxin genes.

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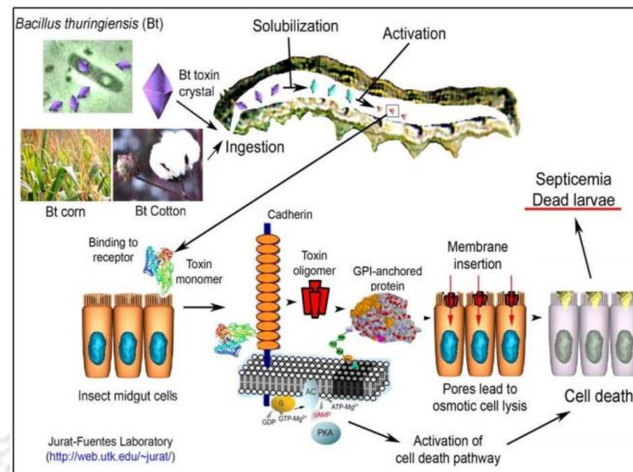

Fig 2.6 **Mode of action of Bt crystalline proteins** (Source: http://web.ulk.edu/~jurat)

A first decisive step in this direction was taken in 1987, with the production of transgenic tobacco expressing *Btcry1Ab* gene (Vaeck et al. 1987) targeted against the European corn borer (ECB), one of the main pest attacking maize in the US and Europe. In starting, to express Bt cry genes in plants, the bacterial coding regions was simply placed between highly active promoter functional in plants and a region providing transcriptional termination and polyadenylation functions (Barton et al. 1987; Fischoff et al. 1987). Mostly, the promoter was the region of cauliflower mosaic virus responsible for the transcription of the abundant 35S RNA (Ali et al. 2006). Transgenic generated by this approached showed very poor expression of toxin and too weak for protection against insect predation (Ali et al. 2006; Koziel et al. 1993). Protection was then improved when only the toxic N-terminal of the protein was expressed in plants (Fischoff et al. 1987), but the protein level was still relatively lower. Then Vaeck et al (1987), achieved a substantial increase in Bt gene expression levels by exploiting a coupled selection system that linked the expression of an antibiotic resistance gene. The transformed plants were selected for high levels of antibiotic resistance, and then co-selected for high levels of expression of the Bt gene. During the 1990s, researchers made a significant breakthrough in the expression levels of Bt genes (Perlak et al. 1990; Wunn et al. 1996;

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Cheng et al. 1998; Khanna and Raina 2002). Unlike plant genes, Bt genes have a high A+T content (66%), which is a suboptimal codon usage for plants, and potentially leads to missplicing or premature termination of transcription (Dela Riva and Adang 1996). The coding sequence of cry genes has been modified (without modifying the encoded peptide sequence) to ensure optimal codon usage for plants, and this allowed toxin production in plants to be increased by two orders of magnitude (Perlak et al. 1991).

2.12 Conclusion and Future perspectives

J. curcas is a valuable multipurpose plant to alleviate soil degradation, deforestation and for bio-diesel production besides valuable by-products and environmental protection (Islam et al. 2011). The unsubstantiated claims and known facts must be balanced to have an objective view on the chances of this plant delivering its perceived potential (Jongschaap et al. 2007). Evaluation of *J. curcas* accessions in terms of their agronomic performance and yield suggests that the optimization of simple agronomic practices and breeding can help to deliver quantifiable increases in yields. However, conventional plant breeding approaches are lagging behind due to the unavailability of systematic survey on the genetic variability *J. curcas* accessions. Therefore, for improvement of this species widening the genetic base through the introduction of accessions with broad geographical background and assessment of fuel properties, life cycle analysis, selection of elite genotypes and development of resistant varieties are necessary (Johnson et al. 2011; Mukherjee et al. 2011). A combination of systematic study of genetic diversity and assessment of fuel property, environmental impact assessment and utilization of transgenic approaches along with plant breeding will be useful for developing elite varieties with desirable yield for strengthening the energy security and protecting the environment from poisonous emission of fossil fuel while greening and reclaiming the wasteland and marginal land.

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Chapter 3

Screening of seed traits and oil content of *Jatropha curcas* germplasm for identification of elite accession

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3.1 Introduction

Jatropha curcas is a perennial tropical crop native to Mexico and Central America (Sun et al. 2008). The seeds are rich in oil, which upon transesterification can be easily converted into biodiesel meeting international standards (Azam et al. 2005). Besides, the seed as a source of important non-edible alternative fuel, the plants have ability to adapt to wide range of environmental and ecological conditions, and they can survive with minimum farm inputs even in marginal and degraded land (Francis et al. 2005; Jongschaap et al. 2007). These attributes confer *Jatropha* as the most promising biofuel crop attracting attention of many governments, private companies and community project developers all over the world (GEXI, 2008). *Jatropha* was introduced to India and it has adapted well to Indian conditions, growing in many parts of the country. However, success of *Jatropha* seed based biodiesel production programme mainly relies on raising of large scale plantation of high yielding accession. India with its diverse agroecological regions and climatic conditions offers a good opportunity for propagating variation. Therefore, systematic collection, screening of seed traits and oil content could lead to identification of elite accession to be used for national biodiesel programme. Such genetic variation often leads to different agronomic performance under varied environmental conditions. Seed weight (0.4 to 0.7 g) and seed dimensions (15-17 mm length and 7-10 mm width) have been shown to vary greatly depending on the prevailing climatic condition and nutrition of the soil (Martinez-Herrera et al. 2006). The oil content was found to vary in the range of 30-42% (w/w) (Surwenshi et al. 2011). This variation depicts the dominant role of habitat and environmental conditions over genotype in determining phenotypic variations in *J. curcas* (Kaushik et al. 2007). A number of research initiatives have been taken to work on collection and evaluation of native populations of *J. curcas* to understand degree of genetic variation (Openshaw, 2000; Sujatha et al. 2005; Ovando-Medina et al. 2009). Variation in terms of morphology, germination and seedling growth has been reported in *Jatropha* of central

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India (Ginwal et al. 2005) and seed oil trait divergence has been recorded in *Jatropha* collected from different locations of Haryana (Kaushik et al. 2007). The authors reported significant differences (P<0.05) in seed size, 100-seed weight and oil content between accessions and the coefficient of variation was found higher for phenotype than genotype, indicating a predominant role of the environment. Genetic association, variability and diversity in seed characters, growth, reproductive phenology, yield and oil content in *Jatropha* have been studied at the level of provenance from different locations in Andhra Pradesh (Rao et al. 2008). Gohil et al (2008, 2009) studied genetic variability in terms of growth, phenology, seed trait and oil percentage of *Jatropha* accession collected from Gujarat Orissa and Rajasthan. Wani et al (2012) evaluated seven accessions from North India (Jammu and Kashmir State) on the basis of plant phenotype, seed traits and oil content to identify elite accession for commercial cultivation. However, a comprehensive work on collection of germplasm and evaluation of genetic variation in *J. curcas* populations in India in general and Northeast India in particular is lacking. Release of high yielding cultivars is impossible without ascertaining the magnitude of variation in the available germplasm, interdependence of growth pattern with yield, extent of environmental influence on these factors, heritability and genetic gain of the material. The knowledge genetic variation would provide useful information about the plant type and especially will be beneficial for selection of elite germplasm.

North East India is well known for rich biodiversity and *J. curcas* is widely found in this diverse landform. In Assam, a state of North East India, government, has also started large scale plantation in order to enhance livelihood of rural people. However, this large scale plantation holds the risk of unsustainable practices and loss of resources, as there no proper comprehensive study has been conducted for screening and identification of *J. curcas* accessions from North East India for better seed trait and high oil content. In a preliminary work, Saikia et al (2009) studied four accessions from

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Assam and eight accessions from other states of North East India for analyzing genetic variation on the basis of plant phenology. However, they did not consider seed traits and oil content, which are most important attributes for evaluation of any biofuel crop and for screening elite germplasm for commercial cultivation in that region. The present investigation was undertaken to assess the variability in terms of seed traits and oil content among native population of *J. curcas* in Assam, North East India, in order to identify elite germplasm.

3.2 Materials and methods

3.2.1. Seed collection

An exploration trips was conducted during the months of July–Aug, 2009 to collect seeds from various sites representing different agroclimatic zones of Assam in 13 districts including Kamrup, Barpeta, Dhemaji, Darrang, Dhubri, Jorhat, Kokrajhar, Morigaon, Nalbari, North Cachar Hills, Lakhimpur, Sonitpur and Tinsukia (Fig. 3.1). Seeds were also collected from two other states Chattisgarh and Gujarat of India as out-group. The selection was based on extensive phenotypic assessment of qualitative characters.

3.2.2 Measurement of seed morphometric characters

Seeds were separated from the fruit mechanically and washed thoroughly with tap water and then dried in hot air oven under similar temperature and humidity conditions to reach constant weight. Then seed coats were removed and 100 random fresh seeds were taken in three replicates (total 300 seeds) from each seed sample and measured for their length, breadth and thickness in millimetres and weight in grams.

3.2.3. Oil extraction from seed samples

One hundred seeds in three replicates were used to extract oil. The dry, cleaned seeds were ground to powder and from grounded samples oil extracted in the soxhlet apparatus using hexane as per the standard AOAC (American Oil Chemical Society)

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procedure for 8 h. The extract was concentrated in rota vapour and the residual oil was cooled and weighed.

3.2.4. Statistical analysis

The data generated from three replications were analyzed using ANOVA and the genetic parameters such as phenotypic and genotypic coefficient of variation (PCV and GCV), heritability in broad-sense (h^2 in %) and genetic advance as percent of mean (genetic gain) were carried out as suggested by Johanson et al (1955). Correlation coefficients were calculated according to Al-Jibouri et al (1958). Mean data over replications were standardized (mean = 0 and variance =1) and used for principal component and cluster analyses. The standardized data matrix was used to generate distance matrices based on Euclidean distances. Clustering was carried out using the Unweighted Pair Group Method using Arithmetic Average (UPGMA) technique. Analysis was performed using Numerical Taxonomy and Multivariate Analysis System, version 2.0 NTSYS-pc (Rohlf, 2000).

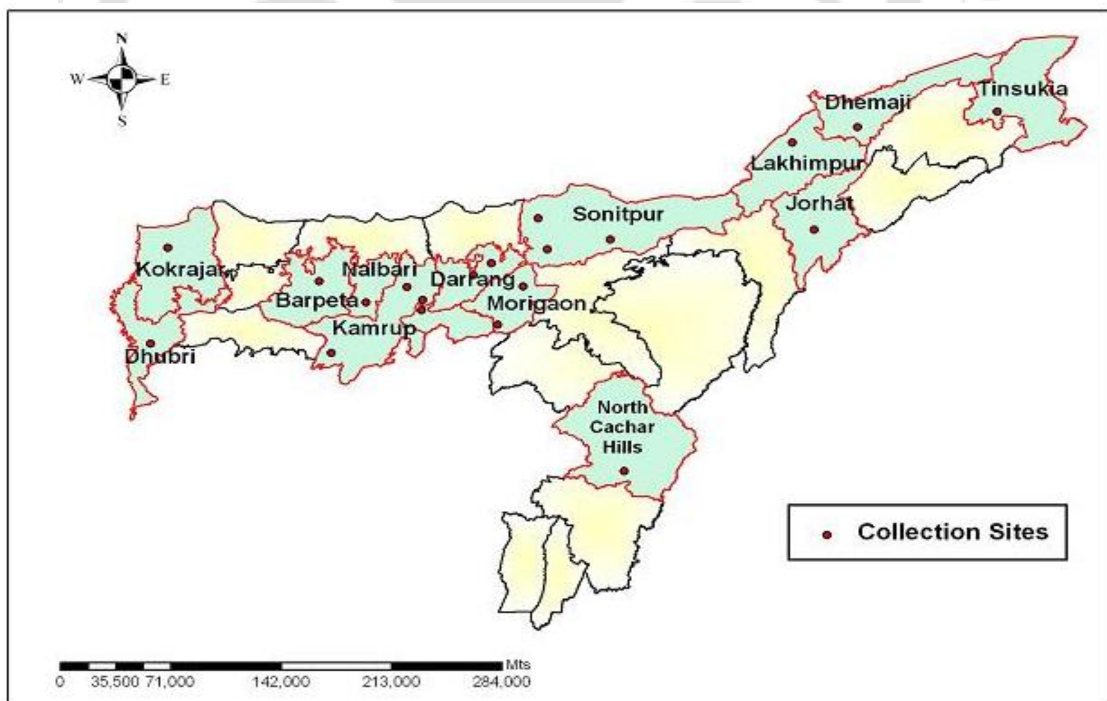

Fig. 3.1 **Map showing the collection sites of *J. curcas* accessions represented in this study**

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3.3 Results and discussions

3.3.1 Seed morphometric analysis and oil extraction

Jatropha seed collected from 20 different sites of Assam (Fig. 3.1), their geographical location and agro-climatic conditions are presented in Table 3.1. This table 3.1, represent altitude, latitude, longitude and annual rainfall as well as annual temperature prevailing in the collection sites. From each wild genotype, 100 seeds were drawn randomly in three replicates (total 300 seeds) for study. Seed coats were removed and morphometric analysis was performed (Table 3.2). Morphometric analysis revealed occurrence of wide range of variability in different attributes of seed. These variations in the seed morphology are likely to occur due to the variation in environmental factors such as rainfall, temperature, relative humidity, and soil types. In natural population, these environmental factors along with biotic factors play a contradictory evolutionary role in governing the seed size (Eriksson et al. 1999). A comparison of mean performance revealed maximum seed length in IITJ13 accession (17.80 mm) and minimum in IITJ20 accession (14 mm). Seed breadth ranged from 7.8 (IITJ20) to 11 mm (IITJ11). Seed thickness varied from 4.60 mm (IITJ20) to 8.00 mm (IITJ14). Hundreds seed weight varied from 36.57 to 56.77 g and highest seed weight observed in IITJ11 accession. Seed length and breadth analysis carried out for *Jatropha* collected from different locations of Haryana showed variation range from 16.00 to 17.63 mm and 10.16–10.92 mm respectively (Kaushik et al. 2007). On the other hand, accession from different zones of Andhra Pradesh showed seed length variation range from 16.00 to 18.00 mm (Rao et al. 2008). Hundreds seed weight was also reported to vary from 49.20 g to 69.20 g and 56.18 to 77.35g in case of *Jatropha* accession collected from different location of Haryana and Andhra Pradesh respectively (Kaushik et al. 2007; Rao et al. 2008). This variation indicates the extent of divergence among Jatropha accessions grown in different regions. Oil content is the most important trait, which decides the overall commercial value of a biofuel crop. In previous studies, oil content was observed

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to vary from 29.26 % to 38.80 % for Jatropha accession collected from different locations of Haryana (Kaushik et al. 2007), 35 % –40 % for Jatropha accession collected from North Telangana zone of Andhra Pradesh (Sunil et al. 2008) and 29.85 % to 37.05 % from Andhra Pradesh (Adilabad, Chittoor, Cuddapah, Anantapur, Kurnool, Nellore, Prakasam, Guntur, Visakhapatnam, Vizianagaram and Srikakulam district). In our study, oil content varied from 23.70% (IITJ11) to 46.60% (IITJ20), which was much higher compared to reported accessions. Among all accession studied, IITJ20 (46.60%), IITJ18 (39.30%), IITJ9 (38.32%) and IITJ8 (37.63%) showed relatively higher oil content depicting promising source biodiesel production.

3.3.2 Genetic variability and association analysis

The extent of genetic variation expressed by different seed parameters and oil content could be analyzed from phenotypic coefficient of variability (PCV) and genotypic coefficient of variability (GCV). Estimates of variability parameters for length, breadth and thickness of seed, 100 seed weight and oil percentage are presented in Table 3.3. The value of PCV is higher than the corresponding GCV for all attributes of seed, in agreement with the previous report of Kaushik et al. 2007 and Rao et al. 2008. The highest GCV and PCV are found for seed breadth followed by oil content. The GCV depends on the heritable part of variability and therefore it is regarded to be more useful for the assessment of inherent or real variability. The PCV values are higher than that of GCV values, indicating the influence of environmental parameter and non-additive gene action on expression of these characters (Kaushik et al. 2007). However, the close correspondence of GCV and PCV values, observed in the present study, suggested the genotypic differences also playing important role in determining variability among the different parameters. Therefore, these characters can be relied upon and simple selection can be practiced for further improvement. These results are in agreements with the findings in Amaranthus (Revanappa and Madalageri 1998), Chenopodium (Bhargava et al. 2003) and *Amaranthus tricolor* (Shukla et al. 2004).

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A high heritability estimate of a character provides a measure of the effectiveness of selection on phenotypic basis for that particular character. Therefore, knowledge of broad sense heritability is very essential as it helps breeders to determine the extent to which improvement is possible through selection by providing index of the relative role of heredity and environment in the expression of various traits (Kaushik et al. 2007). Highest heritability of 99.00% was recorded for oil content closely followed by seed weight in case of accessions collected from Haryana and Andhra Pradesh (Kaushik et al. 2007). However, in our study, broad sense heritability for all seed parameters and oil content are found to be exceeding 97% for all the seed attributes studied among the accessions collected from Assam. Among all characters, seed breadth showed highest heritability (99.57%) followed by oil content (98.82%), seed weight (97.99%) and seed thickness (97.08%). The higher value of broad sense heritability for these characters indicated these characters are the best gain characteristics and therefore, potential target indices for *Jatropha* improvement because of its strong genetic control. However, it should be noted that estimation of heritability is of little significance in coherent selection breeding programs unless accompanied by sufficient genetic gain (Tefera et al. 2003). Genetic gains depend on the type and extent of genetic variability (Kaushik et al. 2007). Due to large differences in the phenotypic variation between different traits, genetic advance is not directly related to heritability values (Rao et al. 2008).

In this study, low to moderate genetic gain for seed and oil trait indicated that improvement could be made in these characters similar to the report of Andhra Pradesh accessions (Rao et al. 2008). Maximum genetic gain was observed in seed breadth (43.55%) followed by oil content (30.66%), seed weight (22.23%) and lowest in case of seed length (12.64%). Maximum genetic gain was observed in seed breadth implicating the additive gene action and existence of a wide range of variability.

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Table 3.1 Geographical locations (latitude, longitude and altitude) and overall agro-climatic conditions (annual rainfall and annual temperature) of the different collection places of *J. curcas* represented in this study

Sl. No.	Sample No	Collected From	Latitude	Longitude	Altitude (m)	Annual rainfall (mm)	Annual Temperature °C (min-max)
1	IITJ1	Kamrup-1 (Amingaon)	26° 16' 60N	91° 40' E	31.0	1600	7- 38
2	IITJ2	Kamrup-2 (Boko)	25° 58' 60 N	91° 16' 0 E	112.0	2234	7-38.5
3	IITJ3	Kamrup-3 (Changsari)	26° 16' 60N	91° 42' E	34.0	2230	7-38
4	IITJ4	Kamrup-4 (Rangia)	26.47' N	91.63'E	38.0	1900	8-37
5	IITJ5	Barpeta	26° 19'N	91° E	34.0	2290	30-35.5
6	IITJ6	Dhemaji	27° 26' 07" N.	94° 39' E.	104.0	4729	5-39.9
7	IITJ7	Darrang-1 (Kharupetia)	26° 31' 11" N	92° 7' 47"E	36.90	1790	10-30
8	IITJ8	Darrang-2 (Mangaldoi)	26.43°N	92.03°E	36.80	1810	10-30
9	IITJ9	Dhubri (Sadar)	26° 02'N	89° 58'E	35.00	2560	7-36
10	IITJ10	Jorhat	26° 45'N	94° 13'E	91.00	2270	7-28
11	IITJ11	Kokrajhar	26° 24'N	90° 16'E	37.00	3714	10-36
12	IITJ12	Morigaon)-1 (Kopahara)	26°50'N	92° E	60.60	2296	10-38
13	IITJ13	Morigaon -2 (Jagiroad)	26.4° N	92.5° E	60.60	2000	10-37
18	IITJ14	Nalbari (Chamota)	26° 25'N	91° 26'E	41.00	2420	10-35
15	IITJ15	North Cachar Hills (Haflong)	25° 11'N	93° 02'E	512.0	2270	10-30
14	IITJ16	Lakhimpur	27°14'N	94° 07'E	87.00	3350	8-32
16	IITJ17	Sonitpur -1 (Bihuguri)	26° 40' N	92° 42' E	60.00	2393	18.1-27
17	IITJ18	Sonitpur -2 (Dhekiajuli)	26° 42'N	92° 30'E	99.00	1200	16-29
19	IITJ19	Sonitpur -3 (Singri)	26° 36' N	92° 28' 60E	42.00	1210	16-28
20	IITJ20	Tinsukia (Makum)	27° 30' 0N	95° 27' 0E	121.0	2000	7-34
21	IITJ21	Chattisgarh (Bilaspur)	21°37"	83.40 "	262 .0	1430	20-25
22	IITJ22	Gujarat (Rajkot)	22° 17' 11" N	70° 46' 9" E	160 .0	2000	15-36

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Table 3.2 Variability in seed morphology and oil content of *J. curcas* accessions

Sl. No.	Sample No	Seed Length (mm)	Seed Breadth (mm)	Seed thickness (mm)	100-Seed weight (gm)	Oil content (%)
1	IITJ1	15.80	8.80	7.4	41.62	34.80
2	IITJ2	15.93	9.00	7.2	43.61	36.80
3	IITJ3	16.50	8.97	6.85	48.99	34.80
4	IITJ4	14.30	8.60	7.23	46.62	30.23
5	IITJ5	16.50	9.00	7.50	38.94	34.17
6	IITJ6	15.00	8.00	6.0	50.90	27.87
7	IITJ7	15.00	9.00	5.83	54.40	36.91
8	IITJ8	14.67	9.00	5.67	53.97	37.63
9	IITJ9	15.50	9.00	7.00	48.99	38.32
10	IITJ10	15.60	9.00	600	52.30	34.33
11	IITJ11	15.00	11.00	6.33	56.77	23.70
12	IITJ12	15.16	9.00	7.20	45.60	32.49
13	IITJ13	17.80	9.40	7.80	48.91	33.27
14	IITJ14	16.60	9.00	8.00	45.60	36.03
15	IITJ15	16.40	9.00	7.02	50.30	28.87
16	IITJ16	15.00	10.00	7.00	56.12	35.13
17	IITJ17	16.83	10.00	7.00	50.09	30.93
18	IITJ18	16.00	9.00	7.50	50.37	39.30
19	IITJ19	16.67	9.67	7.17	50.11	35.17
20	IITJ20	14.00	7.80	4.60	36.57	46.60
21	IITJ21	16.00	9.00	6.33	45.33	32.60
22	IITJ22	14.00	9.67	7.77	42.23	23.80
C.V.		1.84	2.42	3.57	2.70	2.82
SE (±)		0.17	0.12	0.14	0.75	0.55
C.D. 5%		0.48	0.35	0.40	2.14	1.58
C.D. 1%		0.64	0.47	0.54	2.87	2.11

Low genetic advance with high heritability in seed length suggested the predominance of both additive and nonadditive genetic advance in expression of this character.

Table 3.3 Analysis of variance for seed morphometric traits and oil content in *J. curcas*

Traits	Variance		Coefficient of variation (%)		Heritability (h2)	Genetic advances as % of mean
	Genotypic	Phenotypic	GCV	PCV		
Seed length	0.95	0.98	6.22	6.32	97.15	12.64
Seed breadth	3.43	3.44	21.19	21.23	99.57	43.55
Seed thickness	0.66	0.68	11.88	12.06	97.08	24.12
Seed weight	27.56	28.12	10.90	11.01	97.99	22.23
Oil content	25.94	25.64	14.98	15.07	98.82	30.66

The estimates of phenotypic and genotypic correlation coefficients (Table 3.4) showed that the genotypic correlations were higher in magnitude than the corresponding phenotypic ones for most of the character combinations, establishing

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predominant role of heritable factors. Seed length was positively correlated with seed breadth and seed thickness, while seed breadth showed positive correlation with seed weight. However, oil content showed no significant level of correlation with any attributes of seeds, which is contrary to the report of Haryana accessions, which showed positive correlation between seed weight and oil content (Kaushik et al. 2007). This observation, revealed that variation in oil percentage among different accessions of Assam is independent of morphological characters of the seeds.

Table 3.4 Genotypic (G) and Phenotypic (P) correlation coefficient between physical attributes of seed and seed oil content in selected *J. curcas* accessions

Characters		Seed length	Seed breadth	Seed thickness	Seed weight	Oil (%)
Seed length	G		0.447**	0.525**	0.044	0.029
	P		0.442**	0.476**	0.036	0.023
Seed breadth	G			-0.135	0.44**	0.262
	P			-0.131	0.423**	0.249
Seed thickness	G				-0.131	-0.340
	P				-0.128	-0.326
Seed weight	G					-0.271
	P					-0.269

** Significant at 1% level

3.3.3 Genetic divergence studies for seed characteristics and oil content

Analysis of genetic diversity among collected germplasms of *J. curcas* by using non-hierarchical Euclidian cluster analysis revealed the relative contribution of different characters like seed morphology, seed weight and oil percentage to the total diversity and genetic relatedness among them (Table 3.5).

The analysis of genetic diversity of a species helps to understand its evolutionary trend in natural population. Genetically distant parents can be selected for hybridization for obtaining desirable recombination (Tams et al. 2004). Maximum Euclidean distance (7.036) was obtained between IITJ20 and IITJ22, while the minimum distance (0.426) was recorded between IITJJ7 and IITJ8. IITJ22 differed greatly from IIIJ20 in terms of their oil percentage, which could be attributed to their large geographical distance as IITJ20 accession was collected from Tinsukia district of Assam, whereas IITJ22 accession was obtained from Rajkot of Gujarat state. Oil percentage was found highest in IITJ20

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(46.6%) and IITJ22 accession from Gujarat recorded lowest oil content (23.8%). IITJ7 and IITJ 8 showed high similarity in terms of their seed morphology and oil content as their sites of collection were closer to each other as both the collection sites, Kharupetia and Mangaldoi respectively fall under the same district of Assam, Darrang.

On the basis of non-hierarchical Euclidian cluster analysis, all accessions collected from different regions were grouped into different clusters depending on their genetic relatedness as well as divergence. Twenty accessions were placed into a big cluster I, which is subdivided into two sub clusters, IA and IB comprising 12 and 7 accessions respectively (Fig. 3.2). Other three accessions (IITJ11, IITJ 20, and IITJ22) remained separate. PCA plot also supported the clustering pattern obtained from UPGMA analysis (Fig. 3.3). The mean seed length, breadth and thickness obtained for clusters IA are 16.32 mm, 9.15 mm and 7.23 mm respectively. For Cluster 1B, mean seed length, breadth and thickness recorded were 14.95 mm, 8.94 mm, and 6.41 mm respectively. Cluster mean value of 100 seed weight and oil content for 1A was found 46.91 gm and 34.60%, whereas cluster1B recorded 51.58 gm mean seed weight and 33.51% oil content respectively.

Clustering patterns showed a big cluster comprising two sub cluster 1A (12 accessions) and 1B (7 accession) showing genetic closeness among accession and rest three (IITJ11, IITJ 20 and IITJ22) remained separate, showing divergence from the rest as apparent from the PCA plot. IITJ 22 accession collected from Gujarat having minimum seed length (14.00 mm) and low oil content (23.80%) but moderate seed breadth, thickness and weight (9.67 mm, 7.77 mm and 42.23 g) showed divergence from the rest of the accessions may be because of geographical distances from rest of the accessions.

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Table 3.5 Pair-wise Euclidian distance among the *Jatropha* accessions used in the study

	IITJ1	IITJ2	IITJ3	IITJ4	IITJ5	IITJ6	IITJ7	IITJ8	IITJ9	IITJ10	IITJ11	IITJ12	IITJ13	IITJ14	IITJ15	IITJ16	IITJ17	IITJ18	IITJ19	IITJ20	IITJ21	IITJ22
IITJ1	0																					
IITJ2	0.619	0																				
IITJ3	1.699	1.301	0																			
IITJ4	2.012	2.183	2.493	0																		
IITJ5	0.894	1.226	2.056	2.793	0																	
IITJ6	2.94	2.875	2.371	1.927	3.539	0																
IITJ7	3.203	2.789	2.244	2.702	3.896	1.979	0															
IITJ8	3.388	2.991	2.587	2.797	4.109	2.14	0.426	0														
IITJ9	1.658	1.17	1.239	2.078	2.376	2.521	1.837	2.056	0													
IITJ10	2.646	2.27	1.514	2.411	3.239	1.53	0.906	1.255	1.576	0												
IITJ11	4.08	3.991	3.286	2.945	4.582	2.162	2.907	3.113	3.53	2.601	0											
IITJ12	1.274	1.294	1.551	1.001	2.058	1.97	2.37	2.567	1.291	1.884	2.976	0										
IITJ13	2.533	2.372	1.79	3.708	2.34	3.825	3.918	4.294	2.718	3.197	4.203	2.812	0									
IITJ14	1.349	1.249	1.557	2.768	1.446	3.517	3.509	3.798	1.822	2.936	4.263	1.896	1.505	0								
IITJ15	2.151	2.07	1.212	2.277	2.453	1.97	2.677	3.034	2.081	1.865	2.526	1.607	1.94	2.053	0							
IITJ16	2.964	2.629	2.111	2.299	3.673	2.374	1.587	1.851	1.656	1.635	2.449	1.944	3.325	2.889	2.239	0						
IITJ17	2.208	2	1.038	2.77	2.366	2.542	2.788	3.159	2.063	1.985	2.826	1.927	1.504	1.881	0.805	2.326	0					
IITJ18	1.889	1.415	1.314	2.604	2.432	3.11	2.431	2.708	0.854	2.135	3.877	1.764	2.231	1.399	2.167	1.878	2.018	0				
IITJ19	1.907	1.513	0.607	2.729	2.191	2.781	2.522	2.881	1.411	1.869	3.321	1.772	1.455	1.382	1.327	2.049	0.89	1.187	0			
IITJ20	4.628	4.434	5.01	4.94	5.035	4.975	4.314	4.051	4.396	4.53	6.568	4.805	6.547	5.617	5.81	5.437	5.837	5.071	5.436	0		
IITJ21	1.55	1.377	1.145	2.115	1.951	1.856	2.243	2.47	1.626	1.473	3.145	1.378	2.646	2.216	1.507	2.52	1.603	2.153	1.635	4.384	0	
IITJ22	5.102	5.444	5.703	4.442	5.458	4.828	6.097	6.162	5.628	5.818	6.389	4.924	6.364	5.651	5.365	6.117	6.08	5.88	6.083	7.036	5.404	0

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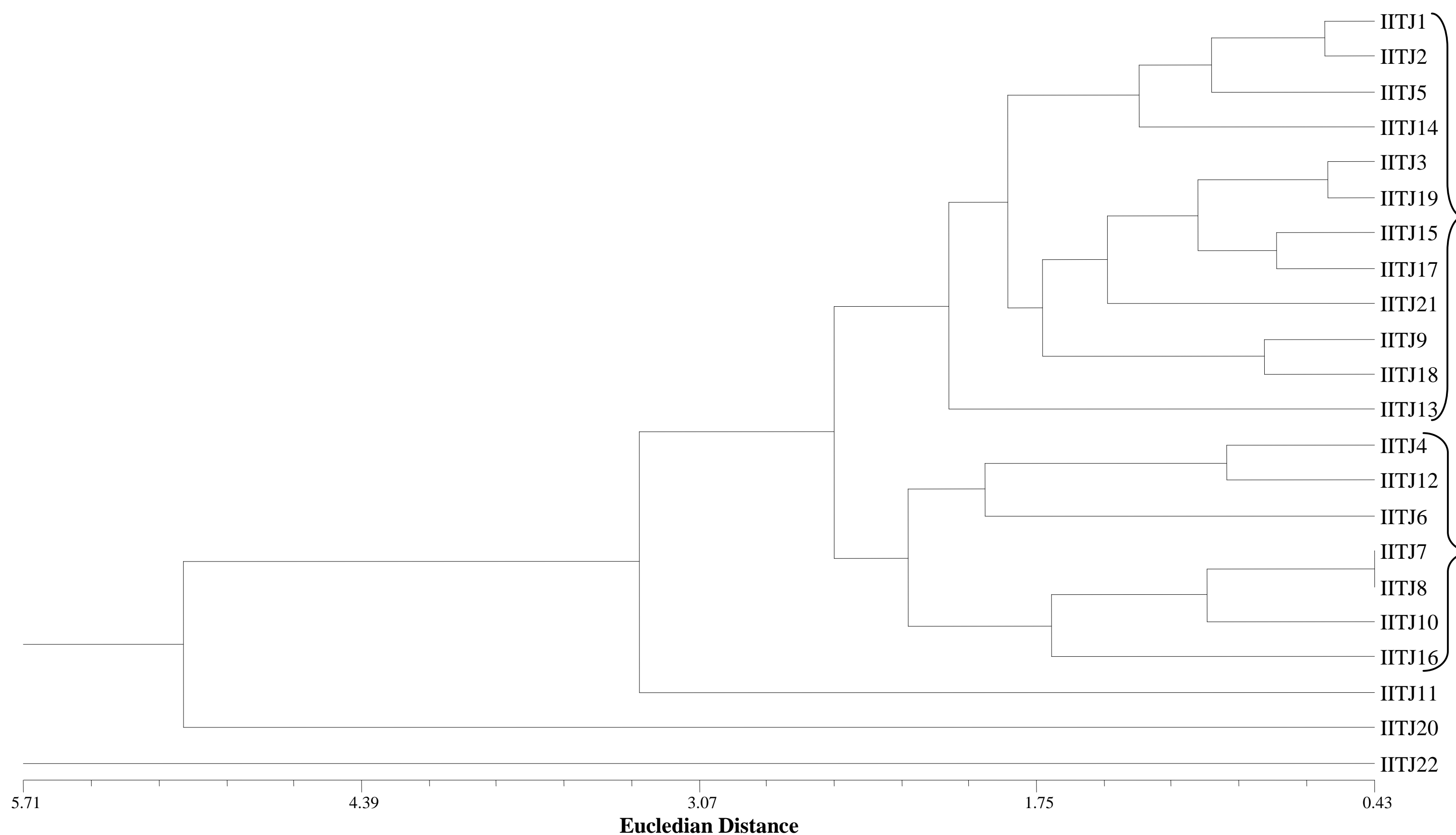

Fig. 3.2 **Dendogram constructed by Unweighted Pair Group Method with Arithmetic mean (UPGMA) of variation in seed traits and oil content in 22 J. *curcas* accessions**

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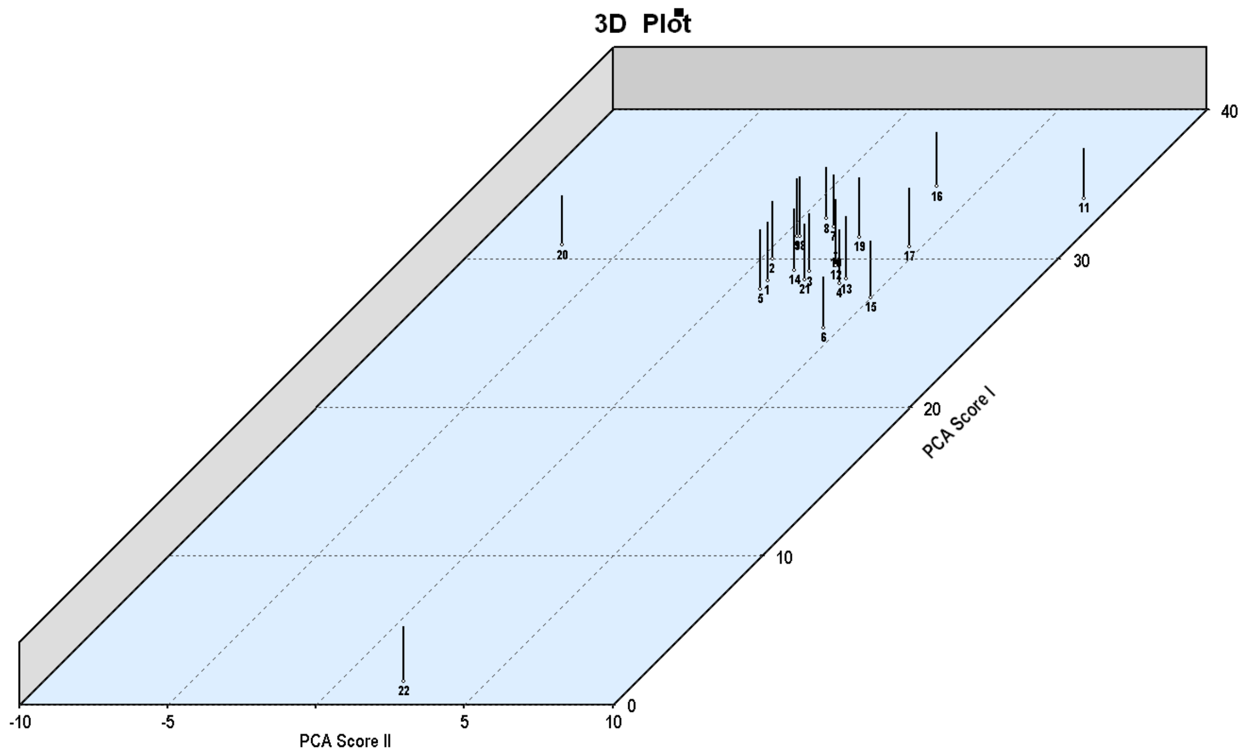

Fig. 3.3 **Principle component analysis of variation in seed trait and oil content in selected *J. curcas* accessions**

However, IITJ21 accession collected from Chattisgarh, which included in sub cluster 1A, showed close resemblance with the accessions collected from different districts of Assam having moderate seed length (16.00 mm), breadth (9.00 mm) thickness (6.33 mm), weight (45.33 g) and oil percentage (32.60 %) implicating geographical distance though important may not be the sole factor in determining genetic divergence. On the other hand, IITJ20 accession collected from Tinsukia, Assam having highest oil content (46.60%) but minimum seed length (14.00 mm), breadth (7.80 mm), thickness (4.60 mm) and lowest seed weight (36.57 g) showed wide range of variation than the rest possibly due to adaptive changes undergone by tress under natural stress and evolutionary forces. Selection of trees as parents from clusters with high inter-cluster distance are useful in developing novel hybrids, whereas, parents from clusters with low inter-cluster distance should be avoided (Rao et al. 2008). Therefore, cross hybridization between IITJ20 accession having highest oil content and

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parent from sub cluster 1A having large seed size may be useful in developing novel hybrid having large seed and high oil content, ideal for commercial energy plantation.

3.4 Conclusion

The present study revealed that *J. curcas* is although well adapted in the unique biogeographic region of Assam, demonstrated variability within the species due to considerable activity of natural evolutionary forces. This variability can be exploited for improvement of this biofuel crop. Cluster analysis based on seed traits and oil content suggested that the accessions could be grouped and such groupings are useful to breeders in identifying possible accession that may be used as parents in breeding for any of the morphological traits included in the present study. IITJ20 (46.60%) was identified as an elite type which can be exploited for commercial biodiesel production while all four high oil yielding accessions, IITJ20 (46.60%), IITJ18 (39.30%), IITJ9 (38.32%) and IITJ8 (37.63%) were advanced for further study to analyze their potential as a source for sustainable biodiesel production.

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Chapter 4

Physico-chemical characteristics of *Jatropha curcas* of Assam for exploration of biodiesel

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4.1 Introduction

Gradual depletion of fossil reserves and emissions of green house gasses are leading to energy insecurity and ecological imbalance in a foreseeable future. Biodiesel derived from vegetable oils is promising as it is renewable, ecofriendly in nature, liquid nature-portability, higher heat content (about 88% of diesel fuel) and lower sulphur and aromatic content (Koh et al. 2011). Furthermore, vegetable oil-based products hold great potential for steering rural economic development because farmers would benefit from increased demand for vegetable oils. Biodiesel is basically monoalkyl esters of fatty acids produced from vegetable oils or animal fats by transesterification with small chain alcohols (Knothe, 2006). The use of biodiesel has grown dramatically during the last few years. Currently, more than 95% biodiesel are produced from edible oil feedstock (Gui et al. 2008). The costs of feedstock account for major percent of the biodiesel production costs including capital cost and return (Bozbas, 2005). Use of less expensive feedstock such as inedible oils, animal fats and waste food oil and by products of the refining vegetables oils is one way of reducing the biodiesel production costs (Veljkovic et al. 2006). However, the availability and sustainability of sufficient supplies of less expensive feedstock will be a crucial determinant to producing a competitive biodiesel. In this regard, inedible vegetable oils, mostly produced by seed-bearing trees and shrubs are good alternative with no competing food uses. Among non-edible oil feedstock, seeds of *Jatropha curcas* proved to be a highly promising reliable source having high seed oil content. The fact that *J. curcas* grows in tropical and subtropical climates across the developing world (Openshaw, 2000), and its seed oil is non-edible due to presence of toxic phorbol esters and curcin makes this crop as fuel source very attractive.

Assam is the gateway state to North East India and it is one of the "biodiversity hotspot" of the world. The species of *Jatropha* is now well naturalized and has become a common flora in this unique bio-geographic region. Recently, government of Assam has initiated state-level *Jatropha* mission for the plantation of this biofuel crop in forest and

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wasteland through forest development agencies, self-help groups and other institutions (http://news.oneindia.in/2006/07/12/assam-government-to-take-up-jatropha-plantation-1152695314.html, http://assamforest.in/extension%20forestry/exten_for.php). The core objective of this programme is to provide employment opportunity to people and to encourage the use of a more environment friendly fuel. Current estimates suggest that there are now 40 hectares of *J. curcas* plantation in Assam with the target of covering additional 2000 acres in near future. However, this large scale *Jatropha* plantation might hold the risk of unsustainable practices and loss of resources. This risk is mainly due to the lack of proper scientific knowledge and technology about the oil content and feasibility of this oil use for production of fuel. However, there is no systematic study made till date on screening and identification of *J. curcas* from North East India for biodiesel production. An investigation on the oil content and physicochemical properties of *J. curcas* in Assam will pave way for judicious selection of *J. curcas* resource for commercial biodiesel production program. In view of this, the present study was carried out to analyze oil content and physico-chemical properties of *J. curcas* collected from different sites of Assam, for exploration of potential biodiesel sources.

4.2 Materials and Methods

4.2.1 Materials and reagent

A through and extensive survey was conducted to collect seeds from different location of Assam as per the procedure described in chapter 3. Four elite accessions (IITJ20, IITJ18, IITJ9 and IITJ9) were selected based on assessment of their oil content (Table 4.1). The seeds were separated from the fruit mechanically and cleaned manually to remove all foreign material. The cleaned seeds were dried under similar temperature (60^{0}C) and humidity conditions to reach constant weight. Pure standards of FAME and Wijs solution (Sigma Aldrich, (St. Louis, MO), and methanol, ethanol, n-hexane, potassium hydroxide, sodium thiosulphate, soluble starch, and phenolphthalein indicator were analytical reagent grade (Merck, Germany).

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4.2.2 Extraction procedure of *J. curcas* seed oil

Grinded kernels were used to extract oil. Oil was extracted in soxhlet apparatus using hexane as per the standard AOAC (American Oil Chemical Society) procedure for 8 h. The extract was concentrated in rota vapour and then cooled and weighed. The physicochemical properties of the oil were determined.

4.2.3 Transesterification of *J. curcas* seed oil

Single step direct transesterification procedure was followed due to low acid value of the native oil. Transesterification experiment was carried out in 250 ml special reaction glass vessels (3 mm thick) sealed tightly and fitted with condenser at the top. The reaction glass vessel was placed on the hot plate magnetic stirrer. Methyl esters of *Jatropha* seed oil were prepared by refluxing the oil at 60°C employing a 1:6 molar ratio of oil to methanol for one and half hour with 1 wt% KOH as catalyst and the mixture was stirred using a magnetic beads (400 rpm) (Darnoko et al. 2000). After completion of the reaction, the mixture was cooled to room temperature and poured in a separation funnel, leading to separation of two phases. The bottom glycerol layer was discarded and the top ester layer was washed gently several times with warm millipore water to remove the trace of catalyst, glycerol, methanol and soap. A pH meter was used to check for the complete removal of the catalyst. The washed methyl ester was further purified under vacuum on a rotary evaporator.

4.2.4 Analysis of *J. curcas* methyl esters (FAMEs)

The fatty acid composition of the methyl esters was determined using a Gas Chromatography (GC, CP-3800, Varian, Netherland) instrument, equipped with a capillary injection system operating at 200°C, with a split ratio of 80:1 and a 1 µl sample size. A fused capillary column (EC Wax with 0.25 mm internal diameter, 30 m length and 0.25 µm film thicknesses) was used. The detection system was equipped with a flame ionization detector (FID). The detector temperature was programmed at 250°C with

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flow rate of 2 ml/min. The carrier gas was high-purity nitrogen gas. The GC analysis was carried out at column temperature program consisted of initial temperature of 160°C, 2°C/min from 150°C to 230°C; increase at 4°C to 250°C and hold for 5 min. The identification of the peaks was achieved by comparing its retention time to the retention time of standards analyzed under the same conditions The percentage of peak area of each fatty acid to the total peak area of all the fatty acids in the methyl ester sample was used to calculate relative percentage of each fatty acid component in the methyl esters. The fuel properties of *J. curcas* oil and the fatty acid methyl esters (FAMEs) were determined as per the ASTM (D6751) standards.

4.2.5 ^{1}H-NMR spectroscopy

^{1}H-NMR spectrum of *J. curcas* oil and the fatty acid methyl esters were obtained on 500 MHz NMR spectrometer (Oxford, AS400, China) using a 5 mm broad band inverse probe head, equipped with shielded z-gradient accessories. Samples were dissolved in 400 µl deuterated chloroform ($CdCl_3$) and transferred to the 5-mm NMR tube. The deuterated chloroform chemical shift peak at 7.26 ppm was taken as internal reference. Typical parameters used were: spectral width: 4,800 Hz; time domain data points: 32 K; flip angle: 90°C; relaxation delay: 5 s; spectrum size: 32 K points; and line broadening for exponential window function: 0.3 Hz.

4.2.6 Thermogravimetric analysis (TGA)

The thermogravimetric profile of *J. curcas* oil and its methyl esters were obtained using Thermogravimetric Analyzer (TGA 851e/LF/1100, Mettler, Switzerland) at the heating rate of 10°C/min in both nitrogen and oxygen atmosphere. The sample size was kept almost same 6±0.5mg throughout the study.

4.3 Results and Discussion

4.3.1 Oil extraction

Geographical location and oil content of four selected elite accession IITJ8, IITJ9, IITJ118 and IITJ20 obtained from the previous study (Chapter 4), was presented in Table 4.1.

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Table 4.1 Geographical locations and oil content of four high yielding *J. curcas* accessions of Assam represented in this study

Accession No	Collection (District)	Oil (%)
IITJ8	Darrang	37.63
IITJ9	Dhubri	38.32
IITJ18	Sonitpur	39.30
IITJ20	Tinsukia	46.60

Among the four accession IITJ20 has highest oil content (46.60%), followed by IITJ18 (39.30%), IITJ9 (38.32%) and IITJ8 (37.63%).

4.3.2 Fatty acid profile of *J. curcas* methyl esters

Fatty acid composition of a biodiesel is an essential parameter for use of the oil as efficient fuels for future generation. Chain length and number of double bonds present in the fatty acid modulates fuel properties of biodiesel. Generally, fatty acids present in a triglyceride are saturated (Cn:0) monounsaturated (Cn:1) and or polyunsaturated with two or three double bonds (Cn:2,3). Vegetable oil having low saturated and polyunsaturated fatty acid was found to more ideal in terms of cold flow properties and oxidative stability. Therefore, vegetable oil higher in monounsaturated fatty acid found as promising alternative to fossil fuels (Gunstone, 2002).

Table 4.2 Fatty acid profile *J. curcas* methyl esters

Fatty acids	Formula	Fraction (%)			
		IITJ8	IITJ9	IITJ18	IITJ20
Palmitic Acid	$C_{16}H_{32}O_2$	12.7	11.5	11.9	11.4
Stearic Acid	$C_{18}H_{36}O_2$	7.9	7.8	7.7	7.8
Oleic Acid	$C_{18}H_{34}O_2$	41.48	44	43.3	46.1
Linoleic Acid	$C_{18}H_{32}O_2$	35.2	31.9	33.1	30.1
Linolenic Acid	$C_{18}H_{32}O_2$	0.3	0.3	0.2	0.3

The fatty acid profile of the methyl esters of four high oil yielding genotypes of *J. curcas* determined by GC were presented in Table 4.2. The main fatty acid components are palmatic, stearic oleic, linoleic and linolenic acid. The fatty acid composition revealed

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domination of mono unsaturated oleic acid followed by polyunsaturated linoleic acid (Table 4.2). The oleic acid content of the oil extracted from the seeds of IITJ20 was higher (46.1%) compared to that of IITJ8, IITJ9 and IITJ18. The oil extracted from IITJ8 seeds showed highest percentage of linoleic acid (35.2%) compared to other three genotypes. The presence of this polyunsaturated fatty acid in the *Jatropha* oil is known to impart semi-drying property and best suitable for use in surface coating industries (Augustus et al. 2002). Besides, oil extracted from the IITJ20 showed higher amount of unsaturation than other three genotypes (Table 4.2). However, the difference in fatty acid composition was found negligible. Therefore, fatty acid profiles of all four accessions rich in oleic acid depicted a promising alternative to diesel fuel in future.

4.3.3 Physiochemical characterization of *J. curcas* oil

The physiochemical characteristics of oils estimated as per the ASTM standard methods were presented in Table 4.3. Initially, the specific gravity of the oil samples was determined using the standard method mentioned as above. Specific gravity of IITJ8, IITJ9, IITJ18 and IITJ20 was found to be 0.868, 0.867, 0.866 and 0.866 respectively, well within the acceptable range of standard ASTM specifications. Similarly, viscosity of oils samples was measured using a standard protocol. Viscosity of oil increased with increase in molecular weight and decreased with increase in unsaturation level and temperature (Nourredini et al.1992). The kinematic viscosity of the oil at (40^{0}C) was found to be 77.40, 46.6, 57.7 and 53.7 cst respectively, an index much higher as compared to conventional diesel (Table 4.3). These high viscosity oils cannot be used directly in engine. High viscosity causes injector fouling and other engine operational problems. Therefore, before application in diesel engine, processing is required to reduce the viscosity of the oil. The flash point of oil samples under study was found to be 310°C, 309°C, 310°C and 308°C for IITJ8, IITJ9, IITJ18 and IITJ20 respectively (Table 4.3). The result showed the flash point of oil samples much higher as compared to conventional diesel. Similarly, the fire point of oil was found at 330°C, much higher than

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diesel. Refractive index is the degree of the deflection of a beam light that occurs when it passes from one medium to the other. The refractive index value increases with the degree of unsaturation. Refractive index of four accessions was found at 1.46. This value indicated the presence of long chain unsaturated fatty acids in *Jatropha* oil. Moisture content of oils was determined using Karl fisher titrator. Moisture content is a qualitative parameter of oil, which influences the storage life of fuel. High moisture content may serve as a medium for microbial growth. Microbial growth in oil may leads to damage of tank and emulsion formation (Monteiro et al. 2008).

Table 4.3 The comparison of physiochemical properties of *J. curcas* crude oil and methyl esters

Properties	IIITJ8		IITJ9		IITJ18		IITJ20		ASTM D6751
	OIL	FAME	OIL	FAME	OIL	FAME	OIL	FAME	
Specific gravity	0.86	0.85	0.86	0.83	0.86	0.83	0.86	0.84	0.860-0.900
Kinematic viscosity at 40°C (cst)	77.40	6.7	46.6	6.3	57.7	6.1	53.7	6.1	1.9-6
Flash point (°C)	310	185	309	185	310	185	308	185	130
Fire point (°C)	330	190	330	190	330	190	330	190	-
Refractive index (27 °C)	1.46	1.45	1.46	1.45	1.46	1.45	1.46	1.45	-
Moisture content (%)	0.31	0.23	0.35	0.25	0.39	0.23	0.22	0.20	0.50 max
Cloud point	11	8	10	8	11	8	11	8	Not specified
Pour point	4	2	4	2	4	2	4	2	-15 to 10
Calorific value(MJ/Kg)	39.80	41.09	39.23	40.27	39.73	40.85	38.31	39.56	-
Acid value (mg KOH g^{-1})	2.24	0.44	2.56	0.44	2.82	0.43	2.90	0.43	0.50max
FFA Content	1.12	0.221	1.28	0.221	1.41	0.218	1.45	0.218	0.25 max
Iodine value	87.56	88.83	111.03	112.30	102.15	100.25	95.17	94.54	120 max

Besides this, it initiates oxidation of oil which effects longetivity of engines and reduced shelf life of oil. The moisture content of oil was found to be 0.31, 0.35, 0.39 and 0.22%

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respectively, well within the acceptable range of standard values (Table 3.4). The flow characteristic of oil was observed under low temperature. The result showed the cloud point 11°C for IITJ8, IITJ18, IITJ20 and 10°C for IITJ9. The pour point of 4^0C was observed for all the four genotypes. The observed cloud point and pour point values of *Jatropha* oil are much higher as compared to conventional diesel. The calorific value for the oil was measured in an oxygen bomb colorimeter (Table 4.3). The data obtained from experiment showed that all four accession having high calorific value in the range of 39.23- 39.80 MJ/Kg (Table 4.3). The acid value of the oil determines the process of transesterfication i.e. either one step or two step process. The acid value of the IITJ8, IITJ9, IITJ18 and IITJ20 was measured to check the free fatty acid content in the oil sample, and it was found to be 2.24, 2.56, 2.82 and 2.90 mg/KOH respectively (Table 4.3). The acid values for all the accessions were found to quite less compare to the reported acid values of *J. curcas* from various other places (de Oliveiraa et al. 2009; Wang et al. 2010; Berchmans et al. 2008). These differences in acid value can be related with the wild origin, maturation stage of seeds and different storage condition (de Oliveiraa et al. 2009). As per the values reported in the literature, FFA content of *Jatropha* oil varied in the range of 5-15% which was far beyond the capacity of conversion to biodiesel via single step alkali catalyzed transesterification. However, the present study, FFA content of oil was found to be very less. Therefore, single step alkali catalyzed reaction was performed for conversion of oil in to biodiesel. This single step transesterification resulted in substantially higher conversion rate and decreased the reagent use and reaction time as compared to two step transesterification process. The high FFA content increased the formation of salts of fatty acids (soap) and conversion rate decreased which caused problem in separation of glycerol at washing step. Iodine value measures the unsaturation content in the oil sample. Iodine values of the oil were found to be 87.56, 111.03, 102.15 and 95.17 g/100 g of IITJ8, IITJ9, IITJ18 and IITJ20 oil sample respectively (Table 4.3).

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4.3.4 Characterization and evaluation of synthesized methyl esters

The fuel properties of methyl esters of four high oil yielding accession determined using the standard protocol was presented in Table 4.3. In the study, it was observed that the specific gravity, which influences the fuel atomization (Canan et al. 2009), was reduced after methanolysis. The obtained values for methyl esters of four accessions were within the acceptable range of ASTM standards (Table 4.3). As described above, the viscosity which was the major problem in the oil samples for engine operation was substantially decreased after transesterification. The decreased value of the viscosity at 40^{0}C was found to be 6.7 cst, 6.3 cst, 6.1 cst and 6.1 cst respectively. The values are almost within the acceptable range of ASTM standards (Table 4.3). Similarly, flash and fire point values were also found to be reduced after transesterification and the obtained values are in the range of 185°C and 190°C respectively. Since, flash point and fire point values are depended on viscosity, therefore, decrease in the viscosity values after transesterification might be one of the reason for reduction. Iodine value of the prepared methyl esters showed almost similar value as that of the oil, that is because this parameter solely dependent on the origin of the oil (Lang et al. 2001). Acid value is another measure of qualitative character of biodiesel. As per the ASTM standard, acid value of transesterified product should not be more than 0.5 mg KOH/g. The acid values of the methyl esters of the four oil samples under the study was found 0.442, 0.442, 0.437 and 0.437 mg KOH g $^{-1}$ respectively, which was below the maximum limits of the standards (Table 4.3). Similarly, the moisture content of the prepared methyl esters samples was determined using Karl fisher titrator. After transesterification the moisture content of IITJ8, IITJ9, IITJ18 and IITJJ20 methyl esters was found to be 0.23%, 0.25%, 0.23%, and 0.20% respectively, well below the maximum acceptable limits. Cold flow properties such as cloud point and pour point of methyl ester was found to be improved after transesterification. Cloud point and pour point of methyl esters was measured as 8°C and 2°C respectively. But these values were still much higher than the conventional

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diesel. The heating value is one of essential property for evaluation of biodiesel, which provides the suitability of fuels as alternative to diesel fuels (Lang et al. 2001). Calorific value for IITJ8, IITJ9, IITJ18 and IITJ20 methyl esters were obtained as 41.09, 40.27, 40.85 and 39.56 MJ/Kg respectively. The result showed that calorific value of methyl esters was higher than the corresponding oils (Table 4). All these properties combined together indicated that IITJ8, IITJ9, IITJ18 and IITJ20 could act as the potential candidates for biodiesel production in North East India.

4.3.5 ^{1}H NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was employed to monitor the transesterification reaction. The use of ^{1}H NMR for monitoring the yield of transesterification reaction was first proposed by Gelbard et al (1995). In case of ^{1}H NMR spectra of methyl esters, signal appear in the region δ 3.7 ppm which indicates the presence methylic esters group (Rashid et al. 1995). ^{1}H NMR spectra was recorded for the oil and as well as for the methyl esters of IITJ8, IITJ9, IITJ18, and IITJ20 accessions. The characteristic peak of methoxy protons observed as a singlet at δ 3.65 ppm and this signal was attributed to methyl esters, which was absent in the oil. Fig. 4.1a and 4.1b represent the ^{1}H NMR spectrum of IITJ8 oil and IITJ8 methyl esters respectively. In case of ^{1}H NMR spectra of IITJ8 oil, multiplet peaks were observed in the region δ 4.11- 4.115, δ 4.266- 4.306 ppm and δ 5.30-5.34 ppm, due to oxymethylic hydrogen that are characteristic of triglycerides from *Jatropha* oil. Fig. 4.1b, ^{1}H NMR spectrum of IITJ8 methyl esters, the strong singlet peak 3.659 ppm was indicative of conversion of parent oil to methyl esters. Therefore, the NMR spectrum of oil and methyl esters, verified successful completion of *Jatropha* oil conversion into biodiesel.

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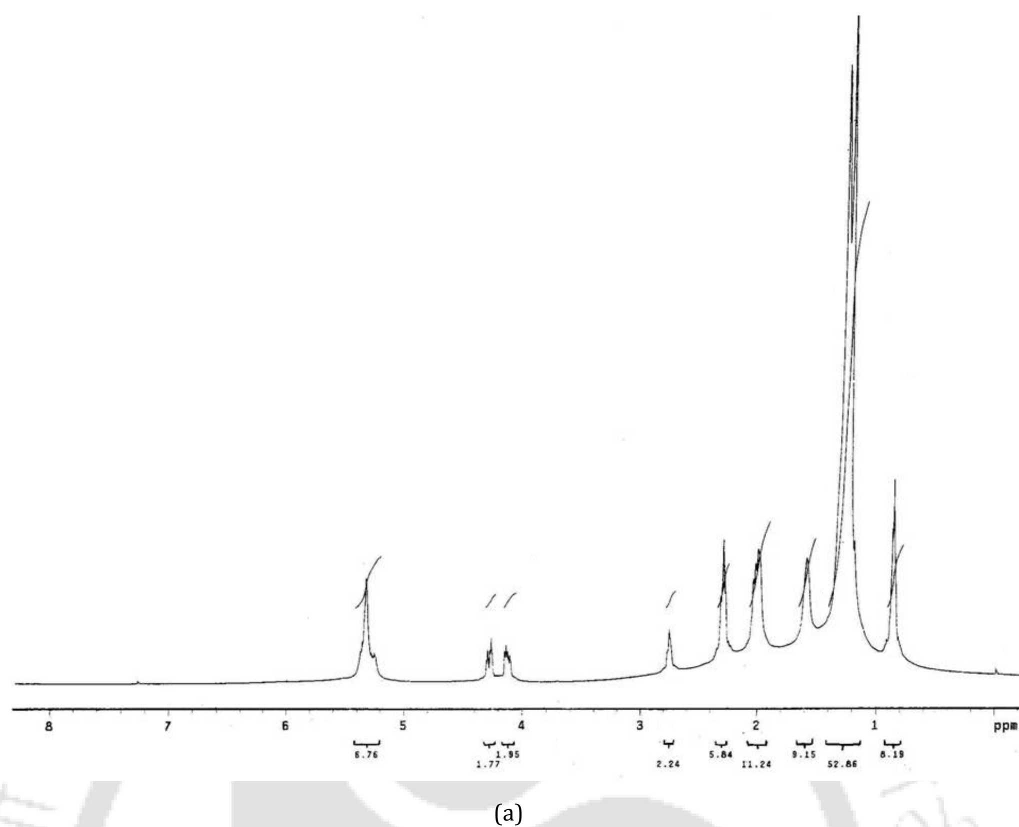

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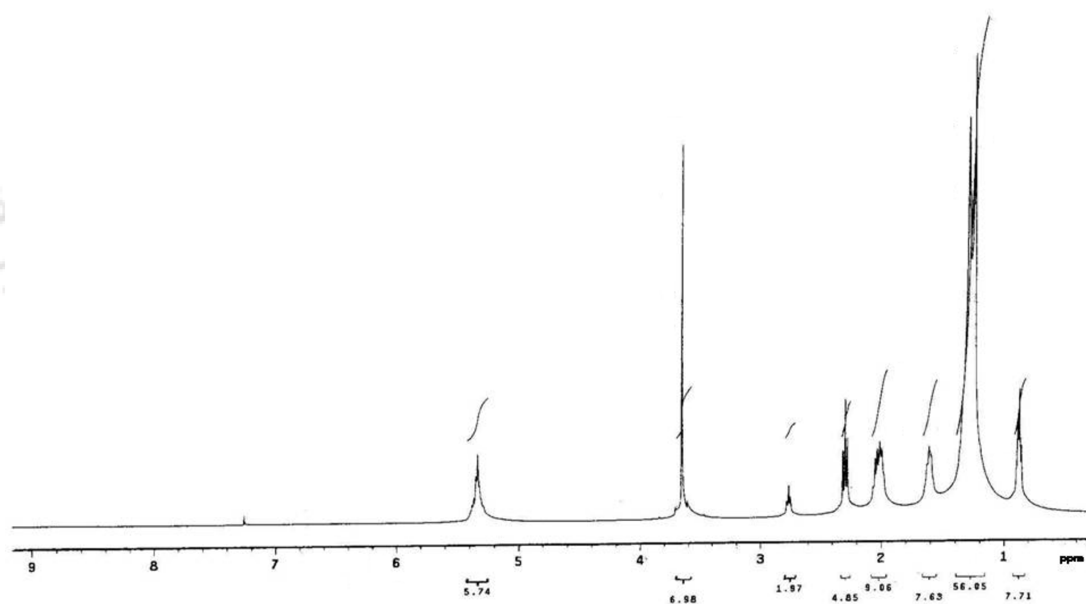
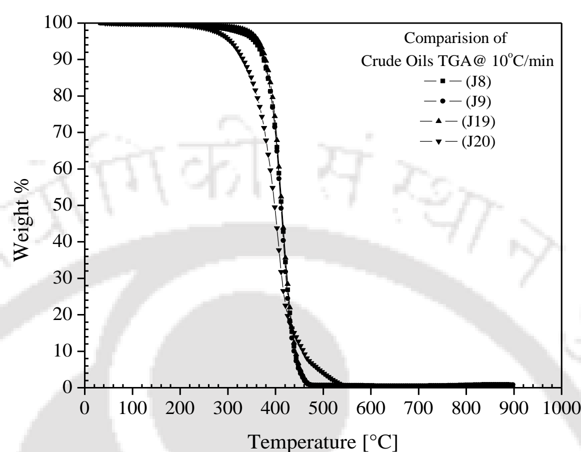


(b)

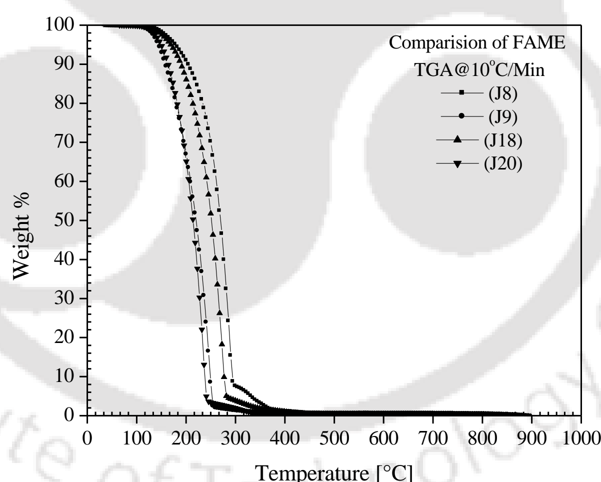
Fig. 4.1 **^{1}H NMR spectrum of *J. curcas* oil and methyl ester** (a) ^{1}H NMR spectrum of crude oil of IITJ8 accession. (b) ^{1}H NMR spectrum of methyl ester of IITJ8 accession, the strong singlet peak at 3.659 ppm is indicative of due to oxymethylic hydrogen is indicative of methyl esters

4.3.6 Thermal stability of *Jatropha* oil and methyl esters

Thermal stability of *Jatropha* oil and its methyl esters was determined from onset temperature of thermal decomposition under nitrogen atmosphere. TGA curve of four oil samples under nitrogen atmosphere is shown in Fig. 4.2.



(a)



(b)

Fig. 4.2 TGA profile of *J. curcas* crude oil and methyl esters under nitrogen environment (a) Comparison of TGA profile of IITJ8, IITJ9, IITJ18 and IITJ20 crude oil (b) Comparison of TGA profile of IITJ8, IITJ9, IITJ18 and IITJ20 methyl esters

The curve showed three consecutive stages of thermal decomposition of the oil samples. Fig. 4.2a depicted the TGA curve of IITJ8, IITJ9, IITJ18 and IITJ20 oil samples. It was evident from the figure that IITJ8, IITJ9 and IITJ18 showing similar decomposition

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pattern. The first stages began at 310°C-315°C, second phase extended up to 470 °C which leads to rapid weight loss. The final stage of decomposition, where pyrolysed product of second phase fully decomposed, extended from 470°C to 900°C. But the TGA curve of IITJ20 showed decomposition at little lower temperature than the other three accessions. In the first stage evaporation started at 240°C-280 °C, extended up to 540° C where rapid weight loss was occurred. Final decomposition occurred at 540°C-900°C. The difference in the decomposition pattern may be due to the presence of less moisture in the IITJ20 oil sample (0.22%) as compared to other three. TGA analysis of methyl esters samples was carried out under similar condition.TGA curve of methyl esters of IITJ8, IITJ9, IITJ18 and IITJ20 samples represented the three stage decomposition at 120°C-125°C, 125°C-360°C and 360°C-900°C respectively (Fig. 4.2b).

From the TGA curves of oils and methyl esters it was observed that, the process of degradation of *Jatropha* methyl esters initiated and completed within a temperature range inferior to the respective oil samples. Molecular tension produced by bulky triglycerides molecule in the oil sample could be the reason for thermal stability of oil (Lujaji et al. 2010). Besides this, high viscosity might be the reason for slow degradation process (Lang et al. 2001). The poor volatility and high viscosity of the oils are the major challenges to run modern diesel engines with plant oils. The onset temperature for volatilization and distillation was calculated from respective TGA curves of *Jatropha* oil and methyl esters and presented in Table 4.4. The result showed onset temperature of thermal degradation of methyl esters was lower as compared to oils.

Table 4.4 Volatilization and distillation temperature obtained from TGA profile of crude oil and methyl esters

Sample name	Onset temperature for volatilization (°C)		Distillation temperature (°C)					
			10%		50%		90%	
	Crude oil	FAME	Crude oil	FAME	Crude oil	FAME	Crude oil	FAME
IITJ8	310	120	390	210	420	280	440	295
IITJ9	310	120	400	160	420	225	450	260
IITJ18	310	120	408	200	425	260	440	280
IITJ20	240	120	350	175	410	220	465	245

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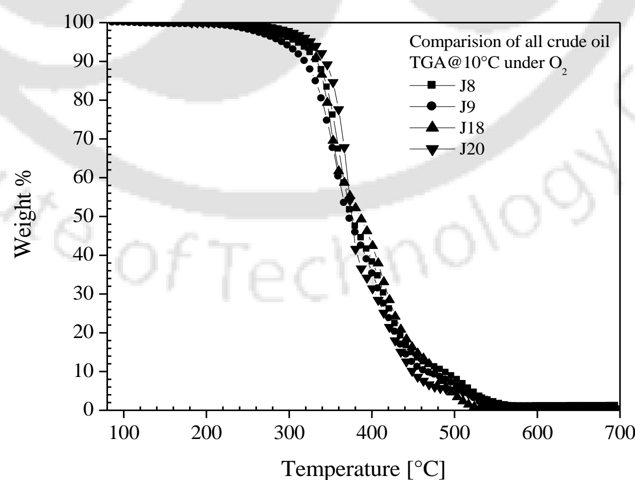
In case of IITJ8, oil sample weight loss was negligible below 300⁰C. But afterwards, rapid degradation was observed at 310⁰C, compared to its methyl esters at 120°C. During this study, it was observed that for all the oil samples 50% weight lost was occurred at around 420⁰C, while in case of methyl esters it was around at 280°C. All the volatile components of the oil which accounted for almost 90% weight were decomposed at around 440°C, whereas in case of respective methyl ester 90% weight lost was observed at 295°C. The remaining 10% was pyrolysis product which was highly viscous liquids, undergoing secondary decomposition. The temperature for secondary decomposition extended up to 470°C-530°C for oil and 320°C-380°C for methyl esters. The residue was completely burnt out after heating up to 900°C for both oil and methyl esters. The data in the table 4.4, confirmed the oil as more stable and less volatile as compared to methyl esters. Further it also confirmed that methyl esters show close proximity with conventional diesel.

4.3.6 Oxidative stability of *Jatropha* oil and methyl esters

Oxidative stability is the quality indicative parameter for methyl esters. It is defined as the resistance of the oil against oxidation.



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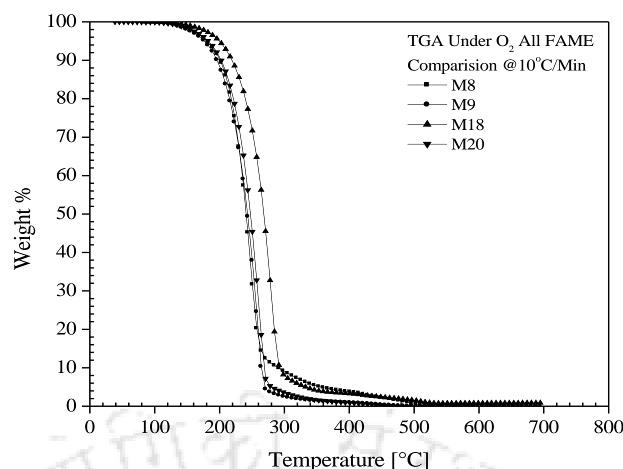

(b)

Fig 4.3 TGA profiles of *J. curcas* crude oil and methyl esters under oxygen environment. (a) Comparison of oxidative stability of IITJ8, IITJ9, IITJ18 and IITJ20 crude oil (b) Comparison of oxidative stability of IITJ8, IITJ9, IITJ18 and IITJ20 methyl esters

The consequence of lipid oxidation results in decreasing the shelf life of oil. The oxidation of long-chain methyl esters initially results in accumulation of hydro peroxides. Then gradually it polymerizes forming insoluble sediments that plugged filters, fouled injectors and interfered with engine operation (Durrett et al. 2008). To measure the oxidative stability of the oil as well as methyl esters, TGA analysis was performed in oxygen atmosphere under same conditions (Fig. 4.3).The Fig. 4.3a show TGA curve for IITJ8, IITJ9, IITJ18 and IITJ20 oil samples under oxygen environment, whereas Fig. 4.3b showed the oxidative degradation curve of their respective methyl esters.

Table 4.5 Onset temperature for oxidative degradation for *J. curcas* oil and methyl esters

Sample name	Onset temperature for oxidative degradation (°C)	
	Crude oil	FAME
IITJ8	230	115
IITJ9	240	115
IITJ18	245	120
IITJ20	240	115

During the analysis it was observed that the onset temperature of oxidative degradation for oil samples was in the range of 230°C-245°C, whereas, in case of methyl esters it was in the range of 115°C-120°C (Table 4.5). That is mainly because methyl esters are less viscous than oil. This low viscosity increases the contact between oxygen and ester molecules resulting higher oxygen diffusion (Ikwuagwu et al. 2000). The vegetable oil contains naturally occurring antioxidants such as tocopherols, sterols and tocotrienols, but the purification process destroys these natural antioxidants and hence becomes prone to oxidation (Sharma et al. 2008). The oxidative stability of methyl ester can be improved either by using synthetic antioxidants which are available in market or vegetable oil based antioxidant additives. It is observed that blending *Jatropha* biodiesel with palm biodiesel improves oxidative stability (Sarin et al. 2007). Further research and development on *Jatropha* based biodiesel will make it more attractive to replace fossil fuels.

4.3.7 Conclusions

Current investigation on oil content and fuel properties of *J .curcas* provided valuable information on potential resources for biodiesel production in Assam. Physicochemical characterization of oil and methyl esters established the suitability of the biodiesel to use in diesel engine. The four accessions used in this study showed low level of FFA, therefore single-step alkali catalyzed transesterification was found to be sufficient for biodiesel production. The present study concluded that four accessions can be used for large scale propagation and cost-effective biodiesel production in North East India. However, more extensive and experimental study needs to be carried out to investigate combustion, emission characteristics and its performance on DI-CI Engine. Therefore, we still need to focus on the process design, and kinetics of *Jatropha* oil transesterification in a batch reactor and analysis in biodiesel-fuelled engine to establish *Jatropha* biodiesel as successful alternative fuel.

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Chapter 5

Life cycle assessment of Jatropha biodiesel production: a case study in North East India

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5.1 INTRODUCTION

Imminent decline of fossil fuel reserves, escalating fuel prices and environmental impact have pinned great hope on biodiesel. Biodiesel is mono alkyl ester derived from plant oils. Currently, about 95% of the world biodiesel production is met by edible oil. Use of edible oil blurred the line between food and fuel economies as both are competing for the same oil resources (Gui et al. 2008). Due to debate on escalating food prices, focus has been shifted to non edible oil sources specially *J. curcas* due to its high oil content, which is prefered in the biodiesel industry as it can reduce the production cost (Chhetri et al. 2008). The close proximity in fuel properties of *Jatropha* biodiesel with diesel and adaptability to diverse land in sub humid to semiarid climates makes this oleiferous crop as one of the best replacement option for fossil fuel. In addition, *Jatropha* derived biodiesel is renewable, biodegradable and shows lower emissions (CO, SOx, HC and particulate emission) in combustion process compared to diesel (Rao, 2011). However, the production and utilization of biofuel also have impacts on the environment. It is essential to evaluate their environmental impact in order to check sustainability of a potential fuel. A comprehensive tool for evaluating environmental issue is Life cycle assessment (LCA). Life cycle assessment is a method for assessing the impact of a certain product or service on environment over the whole production phase and usage – starting from the extraction of raw material till the end use of the product (Lindfors et al. 1995). By inventorying all inputs compiled from the production and utilization of the product, obtained output and assigning them to special categories of environmental impact, environmental performances can be calculated (Achten et al. 2008). Several studies have been conducted using this methodology to analyze the environmental load of plant based biodiesel such as green house gas emission (GHGs), carcinogens, acidification, eutrophication, ozone layer depletion, mineral and fossil fuel depletion, land use etc in different parts of the world (Halleux et al. 2008; Hu et al. 2008; Panichelli

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et al. 2009; Yee et al. 2009; Spirinckx et al. 1996; Yu et al. 2009; Luo et al. 2009; Requena et al. 2011; Nazir et al. 2010; Prueksakorn and Gheewala 2006; Pandey et al. 2011).

Several groups of researchers have performed life cycle analysis on different aspects of *Jatropha* biodiesel. Reinhardt et al (2007) performed a screening LCA examining the advantages and disadvantages of *Jatropha* biodiesel in India and compared with conventional diesel system. Environmental impact categories such as energy utilization, greenhouse gas (GHG) emissions, acidification, eutrophication, summer smog formation potential, and nitrous oxide ozone depletion potential were evaluated. The study determined that *Jatropha* cultivation and processing make maximum contribution to net GHG emissions, while minimal contribution by material transportation. Energy consumption and greenhouse gas (GHG) emissions from *Jatropha* Methyl Ester (JME) production in Thailand were analyzed by Prueksakorn and Gheewala (2006) using a life cycle approach. Main contributors to the energy use were found to be transesterification, irrigation, followed by fertilization process. The maximum global warming potential was observed from the production-use of fertilizer and irrigation process. GHG emissions were found 77% lower from *Jatropha* methyl ester, compared to the production and use of diesel. Sampattagul et al (2007) developed the life cycle inventory database of *Jatropha* bio-diesel and analyzed the environmental impacts by using life cycle approach. The analysis showed that the cultivation process of *Jatropha* contribute to the highest environmental impact compared with other stages in the life cycle, as the management of cultivation was not effective enough. Whitaker et al (2009) evaluated the potential for *Jatropha*-based biodiesel in achieving sustainability and energy security goals. The life cycle impact of substituting petroleum diesel with biodiesel blends in Indian locomotives was also estimated. The life cycle of *Jatropha* biodiesel examined in the study included *Jatropha* cultivation, oil extraction, base-catalyzed *Jatropha* oil transesterification to biodiesel, and combustion of blends of 5%

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(B5), 10% (B10), 20% (B20) and 100% (B100) biodiesel in Indian locomotives. The result obtained suggested that substituting petroleum diesel with biodiesel blends yields reductions in both net GHG emissions and petroleum consumption. Substituting petroleum diesel with B5 was found to reduce net GHG emissions by 3%, by 12% for B20 and for reference, by 62% for B100. The life cycle stages that made the greatest contribution to net GHG emissions for biodiesel blends were locomotive operation, petroleum diesel production and distribution and *Jatropha* cultivation. Achten et al (2010) evaluated the life cycle energy balance, global warming potential, acidification, eutrophication and land use impact on ecosystem quality for a small scale, low-input *Jatropha* biodiesel system established on wasteland in rural India and compared with fossil fuel. The analysis of data showed that the production and use of *Jatropha* biodiesel decreases non-renewable energy requirement and reduces global warming potential (GWP) compared to the reference fossil-fuel based system. However, increase in acidification and eutrophication was found higher from the *Jatropha* system relative to the reference case. Fertilizer application (mainly N) was found as important contributor to most negative impact categories. Optimizing fertilization, agronomic practices and genetics are the major system improvement options. Nazir et al (2010) showed that *Jatropha* is superior feed stock then palm by comparing life cycle inventory database of palm and *Jatropha* biodiesel and analyzing the environmental impacts by using the concept of life cycle. The result obtained showed that, the cultivation process contribute highest environmental impacts compared with other stages in the life cycle and biodiesel production from palm oil consume much higher fossil-based energy than *Jatropha* oil. The highest fossil-based energy consumption was in the transesterification process, followed by the plantation and oil extraction. Considerable variation was observed in the estimates of energy and GHGs associated with biodiesel production. This variation is mainly due to the variation in inventory compound and system boundary

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assumption. A study of environmental impact of biofuel crop cultivation and biodiesel production is important, because it provides information that will enable effective measures to be undertaken so as to improve the GHGs and energy of the system. It will also enable out-growers, biofuel developers, policy makers and development practitioners to have better knowledge to draw realistic plan for large scale *Jatropha* production minimizing the environmental burden and economic risk.

We screened elite germplasm of North East India on the basis of oil content and four high oil yielding accessions from this study were selected, which showed oil content range from 37.63 to 46.6% (Mazumdar et al. 2012). Biodiesel preparation was optimized for those elite accessions and the physicochemical properties analyzed were found to be within the acceptable range of standards specifications of ASTM D6751, depicting a promising source for biodiesel in North East India (Mazumdar et al. 2012). Analysis of the impact of this biodiesel production on environment is essential in order to ensure sustainability of this potential fuel. The present study contained in this section was undertaken with the objective to develop a well -to -tank life cycle inventory database for *Jatropha* biodiesel production process and analyse the environmental impact by life cycle approach. The life cycle of *Jatropha* biodiesel is compared with biodiesel made from seeds of *Pongamia*, another non edible oil yielding tree that grows abundantly in Assam. Pongamia and Jatropha are two such non-edible vegetable oil, which have high oil content and their biodiesels shows close proximity to conventional diesel in fuel properties (Mazumdar et al. 2012; Bobade et al. 2012). Therefore, this analysis was carried out to compare the environmental load created by each step of biodiesel production which can provide the information on superiority of biodiesel source in terms of sustainability. This analysis comprises of all steps starting from agronomy to oil extraction and till biodiesel production.

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5.2. Methodology

This study was conducted under the framework of ISO 14040 standards, which consist of four steps: goal and scope defining, life cycle inventory, life cycle impact assessment and interpretation of the output (ISO, 1997). Firstly, defining the goal and scope involves specifying purpose of the study and setting system boundaries. Structure of LCA studies can vary greatly depending on the target subject and quality of data. Secondly, life cycle inventory encompasses data collection for each unit process of mass and energy flow, as well as data pertaining to emission to the atmosphere. Data can be collected from established life cycle inventory (LCI) databases, primary documentation, academic studies or other high quality, verifiable sources. Thirdly, the life cycle impact assessment phase evaluates potential environmental impacts of the modelled system. It is designed to translate the environmental significance of a system's impacts. The life cycle impact assessment (LCIA) phase takes raw input and output data (such as emissions of carbon dioxide and methane) and converts them to common metrics designed to translate the environmental significance of a system's impacts. The life cycle impact assessment (LCIA) phase takes raw input and output data (such as emissions of carbon dioxide and methane) and converts them to common metrics such as global warming potential (GWP). Finally, the last step involves interpretation, which is an ongoing process of analyzing the results of the LCI and LCIA to identify significant issues and evaluates findings to draw conclusions and suggest steps for mitigation.

5.2.1 Goal and scope definition

The main goal of the study was to assess environmental performance associated with the biodiesel production from *J. curcas*. The scope of this study includes farming *Jatropha* seedling in nursery, plantation and fruit harvesting, seed oil extraction and biodiesel production. Furthermore, biodiesel production from *J. curcas* was compared with *Pongamia pinnata* (reference system). *Pongamia* oil is a non edible oil extracted

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from seeds of *Pongamia pinnata*, a member of Fabaceae family. It is a hardy tree, native to the Asian sub-continent. It is also commonly found in Assam, North East India. *Pongamia* seeds contain 30-35% oil (Gogoi et al. 2008). The fuel properties of *Pongamia* biodiesel showed close similarity with fossil fuel (Bobade et al. 2012a). However, viscoscity and cold flow properties of *Pongamia* biodiesel has been found higher than that of *Jatropha* biodiesel (Mazumdar et al. 2012; Bobade et al. 2012a). For this comparison, all the input data's for *Pongamia* was also taken using similar approaches. Data of labour and transport was excluded, as it found to vary with the places. The functional unit of this study was 1ton of biodiesel production from *Jatropha* and *Pongamia*.

5.2.2. System boundaries

The system boundary used in this study has been presented in Fig. 5.1. The life cycle of *Jatropha* biodiesel production comprises of four major steps. First step is the *Jatropha* plantation and production of seed followed by oil extraction and transesterification of oil for biodiesel production.

5.2.3 Life cycle inventory analysis

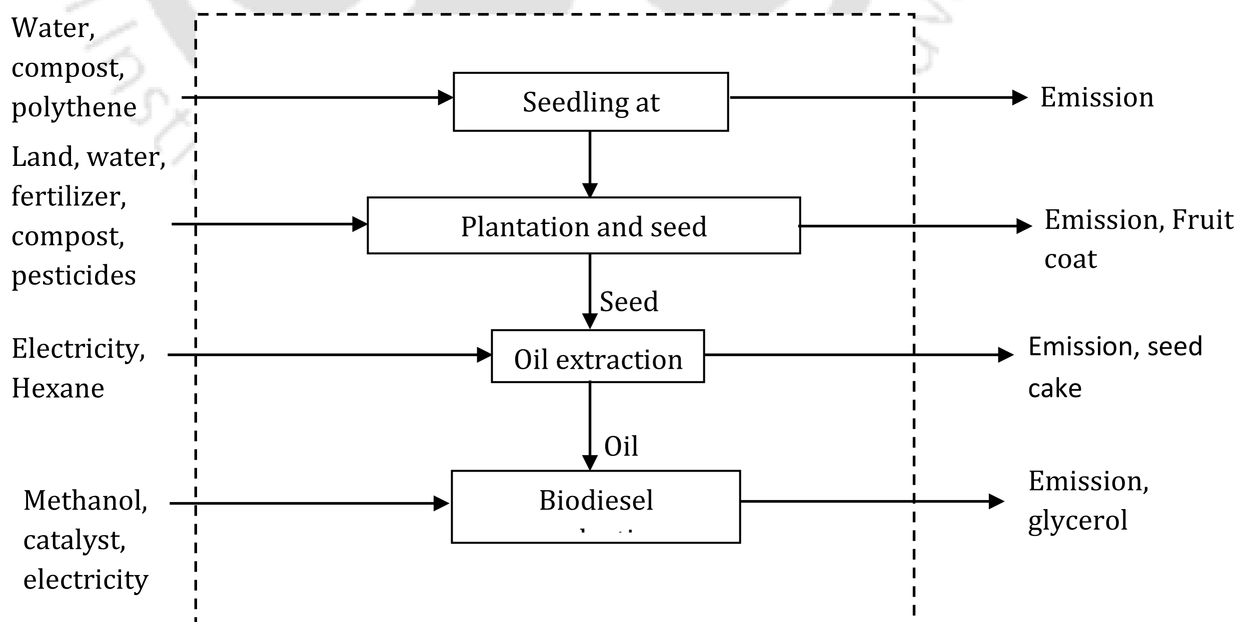

Fig 5.1 **System boundary of the investigation**

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The life cycle inventory analysis was performed on the basis of material and energy inputs and air, water and solid waste emission outputs involved in 1 ton of biodiesel production. Table 5.1, represents input of the various stages involved in 1 ton of biodiesel production.

5.2.4 Process inventory of *Jatropha* oil

Data for *Jatropha* plantation and harvesting were collected from Smiriti Herbs and Biofuel Farm. This farm has been carrying out *Jatropha* plantation since 2006 in the state of Assam, Arunachal Pradesh and Meghalaya under contract Farming.

5.2.4.1 *Jatropha* seedling in nursery

In the nursery, seeds were sown three months before the rainy season in a polythene bag containing soil-sand mixture rich in cow dung slurry and compost. Pots are well watered at regular intervals.

5.2.4.2 *Jatropha* plantation and seed production

At the onset of the rainy season, the seedlings are transferred to the field for plantation. Plantation encompasses several processes including land preparation, watering, spraying fertilizer, pesticides and harvesting. Land preparation mainly consists of land clearing and developing planting pits 30cm x 30cm x 30cm of sizes filled with a mixture of soil, decomposed the cow dung and fertilizer (urea, ssp and mop). The plantation of *Jatropha* has been done in rows at spacing of 2x2 meter accommodating 2500 plants per hectare. Ploughing, harrowing and furrow process were done with tractor containing diesel engine.

The average rain fall of North East India is around 2000 mm, so separate watering is not required for *Jatropha* farming. The NPK in the ratio of 46:48:24 kg per ha are applied from second year of plantation to obtain better growth and economic yield. Pruning and harvesting was done manually. *Jatropha* started fruiting after second year of plantation and from sixth year onward, commercial level of fruiting started. The ripen

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fruits are about 2 – 5 cm long and weight is around 10 to 15 g. Generally 2-3 seeds are found inside fruit and are about 1.1 – 2.0 cm long and weighted 3-5 g each.

5.2.4.3 Oil extraction

Seeds are separated from the fruit manually and then dried under the sun. Average production of seed per hectare of plantation calculated for initial twenty years was 10.1 ton. Generally oil is extracted from *Jatropha* using screw press engines. However, this process leads to low oil recovery (20%-30%) and utilizes considerable horsepower energy and burning of fossil fuels, which causes negative impact on the constant environment. On the top of it, considerable wear happens and maintenance is required. In comparison to that, hexane extraction is relatively efficient and reliable and highest oil recovery was possible (Chalatlon et al. 2011). So, in this study, we have considered hexane extraction for recovery of oil. The input data of oil extraction were obtained from our laboratory experiment (Mazumdar et al. 2012).

5.2.4.4 Biodiesel production

For conversion to biodiesel, alkaline catalytic transesterification process was adopted, as free fatty acid content of *Jatropha* oil was found low in case of North East accessions (Mazumdar et al. 2012). Data related to biodiesel production from *Jatropha* oil were obtained from our lab experiments (Mazumdar et al. 2012). *Jatropha* biodiesel were prepared by refluxing the oil at 60°C employing a 1:6 molar ratio of oil to methanol for one and half hour with 1 wt% KOH as catalyst. The approximated conversion of biodiesel was about 96%.

5.2.5 Process inventory of *Pongamia* oil

Information of *P. pinnata* plantation and their harvesting in North East India were collected from the reported data of Gogoi (2008).

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Table 5.1 Material and energy input used in plantation, oil extraction and production of one ton biodiesel from *J. curcas* and *P. pinnata*

Input types	*Jatropha curcas*	Unit	*Pongamia pinnata*	Unit
Seedling in nursery (1 p)				
Water unspefied natural origin	1250	litre	1250	litre
Compost	0.63	kg	0.63	
Polyethylene, LLDPE, granulate	3.5	g	3.5	g
Plantation				
Jatropha seed production	1	ton	1 ton	ton
Seed production per ha	10.1 ton		3.27 ton	ton
Transformation, from shrub land, sclerophyllous	990.09	m^2	3058.10	m^2
Transformation, to arable, non-irrigated	990.09	m^2	3058.10	m^2
Water, rain	1980.18	m^3	1980.18	
Carbon dioxide, in air	1976	kg	1220	kg
Jatropha seedling in nursery	247	p	122	p
Single superphosphate, as P2O5	29.64	kg	-	
Urea	4.94	kg	-	
Potassium chloride, K2O	3.95	kg	-	
Cow dung	287.5	kg	888	kg
Fertiliser (N)	4.56	kg	19.26	kg
Fertiliser (P)	4.75	kg	17.43	kg
Fertiliser (K)	1.98	kg	11.93	kg
Herbicides	1.36	kg	-	
Diesel	9.53	kg	29.682	kg
Oil extraction				
oil	1	ton	1	ton
Seed taken	2.17391	ton	3.226	ton
Hexane	6.52	ton	19.36	ton
Electricity	73.84	MJ	109.57	MJ
Biodiesel production				
Jatropha biodiesel	1	ton	1	ton
Jatropha oil	1.0416	ton	1.11	ton
Methanol	209.56	kg	261.81	kg
KOH	9.449	kg	-	
NaOH	-		10.08	kg
H_2SO_4	-		5.04	kg
Electricity	73.84	kWh	78.81	KWh

5.2.5.1 *Pongamia* seedling in nursery

Pongamia is propagated by direct seedlings or by planting nursery raised seedlings. Propagation by branch cuttings and root suckers is also possible. Its seeds can immediately be sown after removing from matured pods and they start germinating after 7 days of sowing. In the nursery, seeds were sown in polythene bags containing

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mixture of soil, sand and cow dung compost and pots were well watered at regular interval.

5.2.5.2 *Pongamia* plantation and seed production

The grown seedlings were transferred to the field for plantation. *Pongamia* is a legume plant that grows to about 15–25 meters in height with a large canopy which spreads wide. Land preparation mainly consists of land clearing and developing the planting pits of sizes 46cm x 46cm x 46cm of sizes filled with a mixture of soil and compost in the field. *Pongamia* seedlings were planted in a row at spacing of 5x5 meter. So, a one ha plot could accommodate 400 plants. Land preparation was done with tractor with diesel engine. Like *Jatropha* plantation, no separate watering was required for plantation. NPK and cow dung compost were applied for obtaining better yield. Spraying of other fertilizers and pesticides are excluded in this study due to lack of the data. Pruning and harvesting was done manually. Fruits setting of *Pongamia* starts from fifth year onwards of plantation. It flowers in April-May and fruits mature in January-February. Each pod bears a single seed and average fresh weight of a matured seed is 1.2 gm. Commercial level seed productions starts from 10 years onwards of plantation (Gogoi, 2008).

5.2.5.3. Oil extraction

Seeds are separated from fruit manually and then dried under the sun. Average production of seed per hectare of plantation has been calculated for initial twenty years to be 3.27 ton. Information about hexane extraction of *Pongamia* oil has been collected from literature (Bobade et al. 2012b). The by product obtained from oil extraction step is the seed cake, which is also rich in nitrogen phosphorus and potassium and can substitute organic fertilizer.

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5.2.5.4 Biodiesel production

For conversion of *Pongamia* oil to biodiesel, two steps catalytic transesterification was opted due to high free fatty acid value (20-30%) of oil (Bobade et al. 2012a). Data of transesterification reaction of *Pongamia* oil were taken from literature (Bobade et al. 2012a). In first step, pretreatment was done for oil to reduce free fatty acid content by esterification with methanol and acid catalyst sulphuric acid within one hour time at 57°C reaction temperature. After that base catalyzed transesterification was performed with 1% sodium hydroxide in 13% distilled methanol for 20 minutes at 65°C. The approximated conversions of biodiesel were found to be around 90%.

5.2.5.5 Data analysis

In order to evaluate the environmental impact of biodiesel production, the impact caused by resources used in each step and emission of wastes from production steps are necessary. This information can be obtained through the use of LCA software. Simapro version 7.3.3 was used for this analysis. Simapro is the most widely used LCA software, which allows to model products and systems from a life cycle perspective. It has many features such as, Intuitive user interface, ISO 14040 LCA's, easy modelling with powerful tools, parameterized modelling, multiple scenario analysis and uncertainty analysis. Modules within the SimaPro model are designed to define material, energy, and environmental inputs and outputs that are required for a specific process within the life cycle. The present investigation was carried out under the framework of ISO14040 standards and eco-indicator 99 method was used for evaluation. Eco-Indicator 99 method includes three types of impact categories (i) Damage to human health, which comprises carcinogenesis, organic and inorganic respiratory effects, (ii) Damage to the ecosystem quality, which comprises ecotoxicity, acidification/eutrophication, global climate change land use and (iii) Resource damage, which includes minerals and fossil fuels utilization.

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5.2.6 Results and discussion

5.2.6.1 Environmental impact generated by different steps of *Jatropha* biodiesel production

5.2.6.1.1 Characterization

The inventory list is the result of all input and output environmental flows of a product system. However, a long list of substances is difficult to interpret thats why a further step is needed known as life cycle impact assessment (LCIA) like characterization and normailzation. In characterization, all the substances are multiplied by a factor which reflects their relative contribution to the environmental impact. Characterization depicts the relative percentages contributed by each step to the total impact of each category studied (Fig 5.2). Among all process, seed production shows highest impact in all categories followed by biodiesel production and oil extraction.

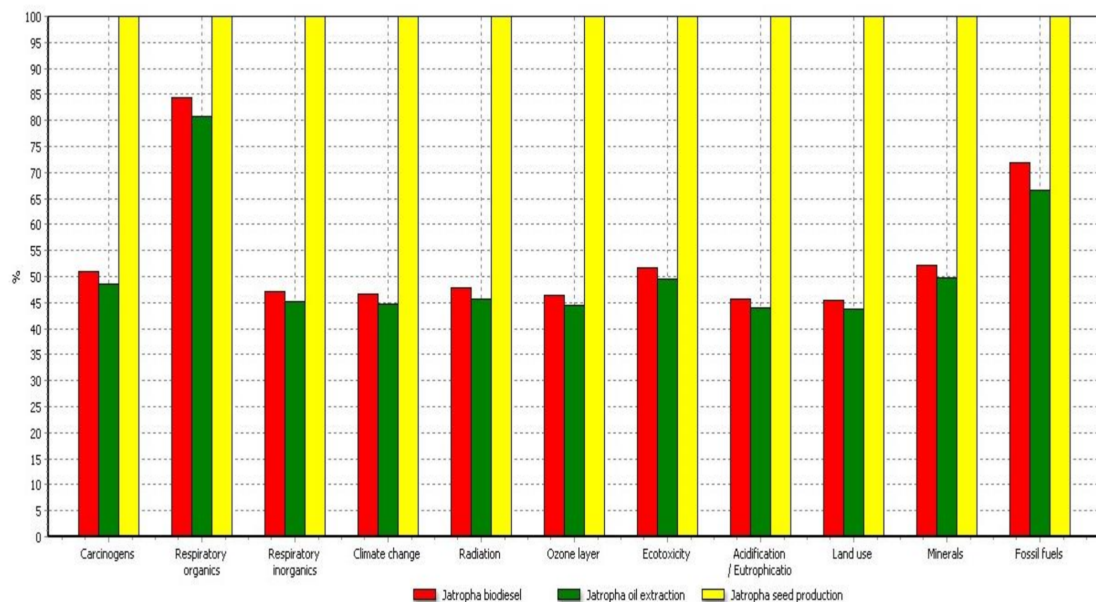

Fig 5.2 **Characterization of different steps of *Jatropha* biodiesel production**

5.2.6.2 Normalization

Through normalization of above output data, we get a clear view of the relative proportion or magnitude of each impact category of a product system under study

(Standard ISO 14040, 2000). It allows monitoring of how the impact category contributes significantly to global environmental problem. Fig. 5.3 represents normalization by impact category of *Jatropha* biodiesel production. In normalization, the most affected impact categories are respiratory inorganics, land use and fossil fuels followed by acidification/ eutrophication, climate change and carcinogens.

Higher value of respiratory inorganics was primarily due to the mineral feedstock like phosphate rock, sylvinite, kieserite and limestone utilized for fertilizer production and chemicals utilized for pesticide production (Gerpen et al. 2000). The utilization of fossil fuel is more in seed production than biodiesel production and oil extraction. Fossil fuel use is more in land preparation and fertilizer production followed by fuel consumption in conversion of oil to biodiesel. This process of fossil fuel utilization has influential role in climate change due to emission of carbon dioxide (CO_2). To some extent, net emission of CO_2 is balanced by CO_2 absorption by plant in plantation. However, emission of methane (CH_4) and nitrous oxide (N_2O) from compost and ammonium fertilizers contribute positive effect on climate change. Carcinogen impact is mainly due to the emissions of arsenic, cadmium and particulate matter. Seed production shows highest acidification/eutrophication followed by biodiesel production and oil extraction. Acidification is mainly caused by nitrogen, sulphur oxides and ammonia which are released during fertilizer application in plantation. Reducing fertilizer rates can also reduce acidification amount. Eutrophication refers to the excessive growth of algae in surface water due to leaching of nitrate and phosphate fertilizers which depletes oxygen content of water ecosystem. The impact of this process can reduce by reducing fertilizer application. Land use impact was also found higher in seed production due to transformation of the land to urban non-irrigated land which entails the use of fertilizers and pesticides which subsequently increases the other environmental loads like respiratory organics and inorganics.

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In categories of respiratory organics along with the seed production, impact of biodiesel production (84%) and oil extraction (80%) was found higher than other impact categories. Ecotoxicity impacts which is found as less effected category compared to other categories mainly occurs due to emission of zinc to air. Among the three steps, seed production showed highest ecotoxicity followed by biodiesel production and oil extraction.

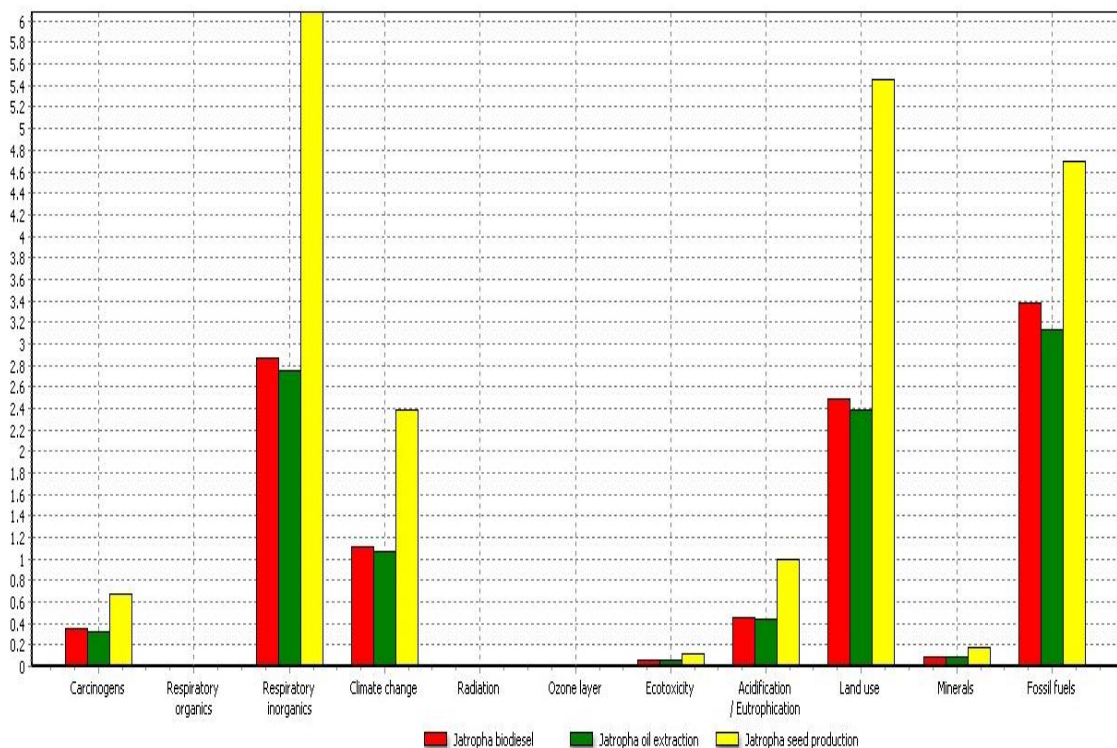

Fig 5.3 **Normalization of the comparison between the production processes of *Jatropha* biodiesel by impact category**

This higher ecotoxicity occurs due to land use and use of fertilizers and pesticides. Combining the effects on all impact categories as a single score showed that seed production showed highest impact in global climate change followed by biodiesel production (Fig. 5.4). *Jatropha* can easily grow in degraded and marginal land. So, in recent years government are focussing on those lands. However, to obtain commercial level of yield in those lands, amount of fertilizer has to use more. This will increase emission of greenhouse gases and particulate matters which will add positive effect on respiratory inorganics, climate change and ecotoxicity.

The by product of oil extraction stage is seed cake of *Jatropha*, which is rich in nitrogen (3.2-4.44%), phosphorus (1.4-2.09%) and potassium (1.2-1.68%) content. Utilization of this seed cake as fertilizer can reduce some environmental load. Besides this, utilization of seed cake as raw material for production of biogas also can reduce environmental impacts. Utilization of fossil fuel in seed production step provides a greater environmental impact followed by biodiesel production. This is mainly due to the use of energy utilized from diesel, natural gas and heavy oils.

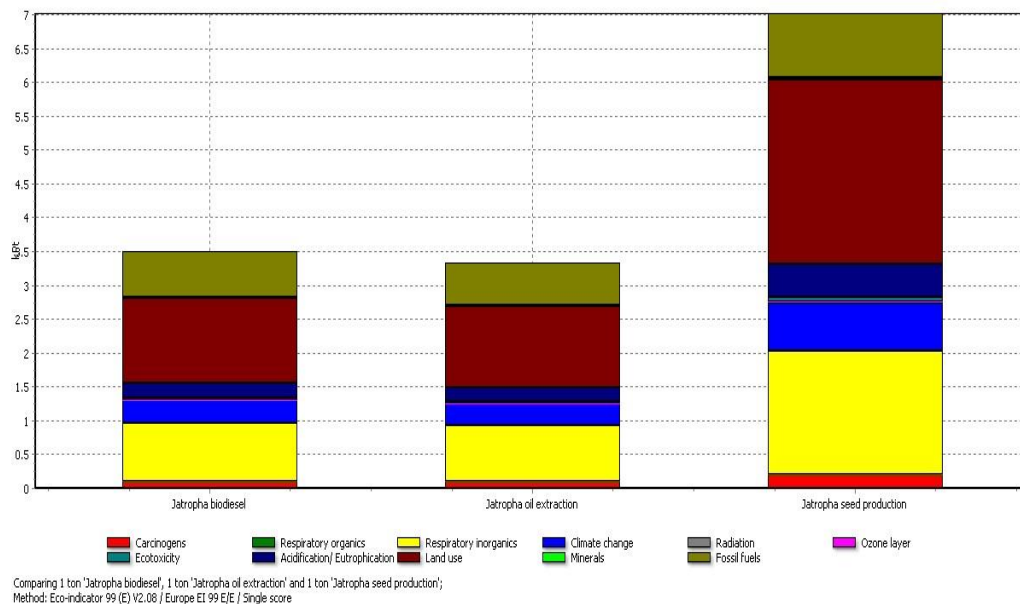

Fig 5.4 **Comparison between the production processes of *Jatropha* biodiesel in single score**

From the above discussion it is clear that among the steps of biodiesel production, seed production showed highest environmental impact. Sincere effort should make to reduce the environmental impact by minimizing the use of inorganic fertilizers and pesticides by planting elite and resistant varieties against biotic and abiotic stresses. Research on genetics of biofuel crop and changes in nutrient cycling as a result of land conversion to biofuel crop will strongly influence the sustainability of plants as energy sources (Davis et al. 2009). So, integration of plant science into the energy industry will be helpful for maximizing biodiesel production along with improving sustainability of climate and soil.

5.2.6.2 Comparison of environmental impact of *Jatropha* biodiesel and *Pongamia* biodiesel

Based on the LCI development from *Jatropha* and *Pongamia* bio-diesel production process in Assam, Fig 5.5, showed the comparison of environmental impacts based on material and energy used in each step of life cycle, *Pongamia* bio-diesel produces higher environmental load than *Jatropha* biodiesel. Among all categories of impact, fossil fuel is the category of most concern followed by land use, climate change and respiratory inorganics.

Most of the fossil fuel input for biodiesel production is due to land preparation and use of natural gas for production of fertilizers and methanol. Average yield of *Jatropha* plantation has been found to be around 10.1 ton/ha/year. For production of 1 ton of *Jatropha* biodiesel would require 990.09 m^2 land per year, whereas, in case of *Pongamia* average yield is 3.7 ton/ ha/year. So, for production of 1 ton of *Pongamia* biodiesel, it would require 3058.103 m^2 land area per year. Therefore, by comparing the land requirement to produce 1 ton of biodiesel, using *Pongamia* is oil as feedstock would require more than double the land area compared to using *Jatropha* oil as feedstock. The requirement of huge land area for *Pongamia* biodiesel is the first indication of unsustainable land area utilization. In addition to that for land preparation more diesels is required and higher amount of fertilizers are used in case of *Pongamia* compared to *Jatropha* which in turn lead to the increase in emission of respiratory in organics and green house gases. Besides this, due to the high free fatty acid value of *Pongamia* oil, two step transesterification are opted, whereas, in case of *Jatropha* oil, one step alkali transesterification was performed. These two step processes consumes additional methanol and sulphuric acid, which increases higher fossil fuel consumption as well as higher global climate change in case of *Pongamia* biodiesel (Fig. 5.5).

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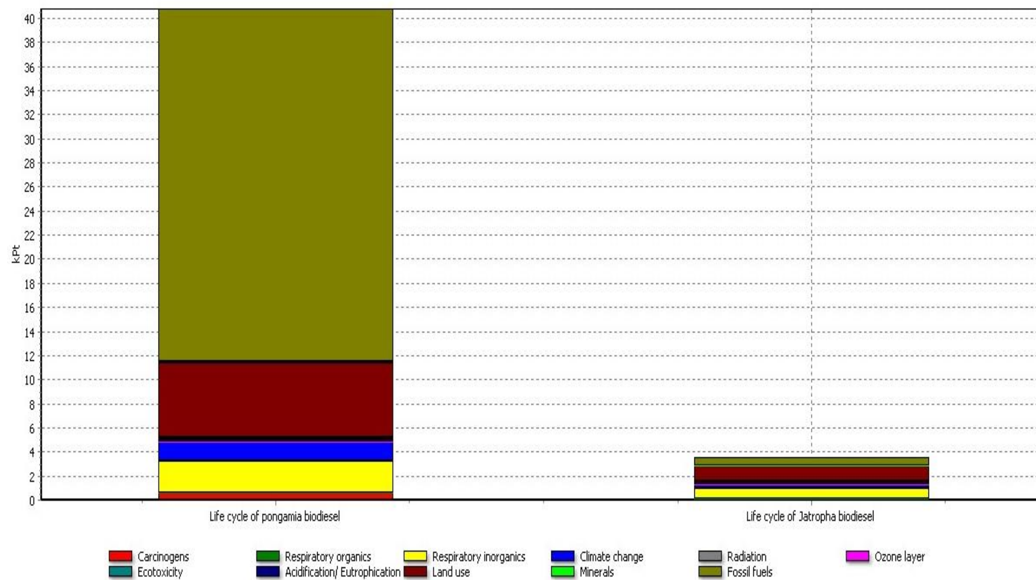

Fig 5.4 **Comparison of environmental impacts of biodiesel production from *Pongamia* oil and *Jatropha* oil based on material and energy used in each step of life cycle in single score**

Combining the effects on all impact categories as a single score (Fig. 5.5), it supports the notion that *Pongamia* biodiesel production (40.2 kpt) generates higher environmental load than *Jatropha* biodiesel production (3.9 kpt).

5.2.7 Conclusion

Of all the processes involved in biofuel production, the cultivation step has greater environmental impact compared with other stages of the life cycle. Specifically, the production and the use of fertilisers have an important contribution to the consumption of fossil fuels, the use of inorganic raw materials, the greenhouse effect and the production of waste. The cultivation process can be improved by using genetically modified varieties resistant to biotic and abiotic stresses and replacing chemical fertilizer with detoxified seed cake.

Biodiesel production from *Pongamia* oil has higher fossil fuel utilization, land use, emission of respiratory inorganics and GHG gases than *Jatropha* oil. Results obtained from comparison of both biodiesel showed that *Jatropha* is a far superior feed stock for biodiesel production as compared to *Pongamia*. The advantages include high

oil content, less land area requirement, simple biodiesel production and low emissions. All these factors ensure sustainability of *Jatropha* biodiesel as a future energy source in North East India. However, effective management of cultivation step, judicial utilization of commercial fertilizer and introduction of genetically modified *Jatropha* varieties resistant to biotic and abiotic stresses may further reduce the environmental burden associated with fertilizers and pesticides and can improve the sustainability of this biofuel crop.

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Chapter 6

Establishment of *Agrobacterium*- mediated transformation system in *Jatropha curcas*

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6.1 Introduction

The utilization of plant biofuel is a promising solution to problems such as the depletion of fossil fuel, fuel crisis and concern over global climate change (Tsuchimoto et al. 2012). *Jatropha curcas* is being promoted as an important non-edible biodiesel crop worldwide. *Jatropha* seed oil which is a triacylglycerol possesses the desired physiochemical characteristics for direct blending with petro-diesel or use as biodiesel after transesterification with methanol (Mandpe et al. 2005). Being a perennial non-edible plant, *Jatropha* offers unique opportunities of both high biomass productions for biodiesel as well preferred source for biodiesel feedstock as it does not compete with production of food crops. However, the demand for biodiesel production is very huge and it cannot be met from wild grown plants. Recently, large-scale *Jatropha* cultivation programs have been initiated in various developing nations including India. Nevertheless, increase of *Jatropha* production requires both increase in area under cultivation and enhance in productivity (Francis et al. 2005). However, low and inconsistent yield, susceptibility to insects and diseases, abiotic stresses including cold and drought (Adebowale and Adedire 2006) and frost (Li et al. 2007) are the major constraints in successful cultivation of *Jatropha* as a bio-fuel crop. Development of superior genotype for resistance to diseases, tolerance to abiotic stresses and high and stable seed yields are the major mandates. Genetic improvement of *Jatropha* for improved traits has been stymied by the narrow genetic variability and limited knowledge of its genetics (Surwenshi et al. 2011).

Genetic engineering over the years has emerged as a powerful tool to complement conventional breeding for improvement of crops including *Jatropha* (Divakara et al. 2010). However, successful genetic manipulation requires establishment of efficient *in vitro* plant regeneration and stable genetic transformation systems (Sharma et al. 2000). Furthermore, an efficient and reproducible genetic transformation

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system would provide a valuable tool for unraveling the functions of genes in *Jatropha* wherein candidate genes involved in fatty acid biosynthesis, biotic and abiotic stress tolerance mechanisms, and regulation of secondary metabolites and phorbol esters, which are considered as important breeding targets, have been identified from whole genome sequencing analysis (Sato et al. 2011), proteomics (Yang et al. 2009) and transcriptomics studies (Costa et al. 2010; Natarajan et al. 2010; Natarajan and Parani 2011). We recently developed a method for rapid and efficient plant regeneration from shoot apices, and generation of transgenic plants by direct DNA delivery to mature seed-derived shoot apices of *Jatropha* (Purkayastha et al. 2010). However, *Agrobacterium*-mediated genetic transformation has become a method of choice for gene delivery, as it offers several advantages such as the defined integration of transgenes, potentially in low copy number, fewer DNA rearrangements and preferential integration into transcriptional active regions of the chromosome as compared to direct DNA delivery (Dai et al. 2001; Travella et al. 2005). There are many reports of *in vitro* regeneration of plants from different tissues of *Jatropha* (Kajikawa et al. 2012). However, stable transformation of *Jatropha* through *Agrobacterium tumefaciens*-mediated approach has been reported using only cotyledon explants (Li et al. 2007; Khemkladngoen et al. 2011; Kajikawa et al. 2012), leaf explants (Kumar et al 2010), and cotyledonary explants (Tsuchimoto et al. 2012). The first report on the establishment of transgenic *Jatropha* from cotyledon explants by *Agrobacterium tumefaciens*-mediated transformation using the herbicide phosphinothricin as the selection agent (Li et al. 2007) was found to result with a very low transformation frequency and not reproducible (Tsuchimoto et al. 2012). Wounding of cotyledon explants by sonication to facilitate *Agrobacterium* infection has been reported to improve transformation (Khemkladngoen et al. 2011) but the process not only found to reduce the post transformation regeneration of shoots but also constrained the root induction (Tsuchimoto et al. 2012). Although successful

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Agrobacterium-mediated transformation of *Jatropha* has been demonstrated very recently (Kajikawa et al. 2012; Tsuchimoto et al. 2012), still the transformation method for this plant is far from routine. In most cases, substantial differences were recorded in the effects of plant growth regulators and optimized selection conditions, suggesting a large variation among different *Jatropha* genotypes and tissues in terms of their response to *Agrobacterium*-mediated genetic manipulation.

One of the key challenges identified in successfully obtaining transgenic *Jatropha* plants is the improvement of transformation efficiency (Tsuchimoto et al. 2012). The present study was carried out to establish a highly efficient *Agrobacterium tumefaciens*-mediated transformation in *Jatropha* by investigating the effects of age and orientation of culture of the cotyledonary leaf segment explants on T-DNA transfer.

6.2 Materials and methods

6.2.1 Plant material and explant preparation

Seeds of a local elite clone of *J. curcas* were collected from Rangia, Kamrup, Assam, India. The seeds were decoated and soaked in distilled water overnight at room temperature. The soaked decoated seeds were treated with a 0.1% sodium hypochlorite solution containing few drops of Tween-20, for 10 min followed by washing under tap water for 20 min. The decoated seeds were then surface sterilized with 70% alcohol for 5 min followed by with 0.2% mercuric chloride for 10 min and finally rinsed four times with sterile double distilled water. After blot-dried on sterile filter paper, the endosperm was carefully dissected out to expose embryos with papery cotyledonary leaves. The papery cotyledonary leaves were separated out and cut into four segments (10 mm^2) with the edges removed and used as explants for callus induction, shoot regeneration and *Agrobacterium*-mediated transformation.

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6.2.2 Shoot multiplication and plant regeneration

The explants were placed with their abaxial surface firmly in contact with MS basal medium (Murashige and Skoog 1962) supplemented with 6.66 μM of 6-benzylaminopurine (BAP) and 0.24 μM of indolebutyric acid (IBA) for 3 weeks in dark conditions for callus induction. The calli were transferred to MS medium supplemented with 6.66 μM BAP, 0.24 μM IBA and 1.44 μM gibberellic acid (GA_3) for induction of multiple shoots. To study the effect of the age of the explants on callus induction and shoot proliferation, and *Agrobacterium*-mediated transformation, the cotyledonary leaf segment explants of different age (cotyledonary leaves obtained from freshly germinated seeds or seedlings developed from embryos on MS medium for 1–2 weeks). To investigate the effect of orientation of explants on callus induction and shoot proliferation, the cotyledonary leaf segment explants of different age were cultured with their abaxial or adaxial surface in firm contact with the medium. The individual shoots were separated from shoot clusters and transferred to MS medium supplemented with varied concentrations of GA_3 (0.5, 1.0, 1.5, and 2.0 μM) for shoot elongation.

MS medium supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar (Hi-Media, Mumbai, India) was used throughout the studies including rooting experiments. The pH of the medium was adjusted to 5.8 prior to autoclaving at 15 psi and 121 °C for 20 min. All the cultures were maintained at 25±2°C under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 35 μmol $m^{-2}s^{-1}$ provided by cool white fluorescent tube lights (Philips, India). After 3 weeks of incubation in the dark, the efficacy of each variant on callus induction and after 4 weeks of incubation of calli in 16 h photoperiod, the shoot multiplication rate were determined by recording (1) the percentage of explants forming callus, (2) the frequency of shoot regeneration and (3) the average number of shoots per callus.

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6.2.3 Rooting and acclimatization

Elongated shoots (2.73-2.75 cm) were transferred to half strength MS medium supplemented with varied concentrations (1.0, 2.5, and 5.0 μM) of IBA and α-naphthalene acetic acid (NAA) for rooting. Plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water and transferred to plastic pots containing soil, vermiculite and vermicompost (1:1:1). For acclimatization, pots were covered with transparent plastic bags to maintain adequate moisture for a week. The plastic bags were removed after 2 weeks and plantlets were maintained in the greenhouse in plastic pots containing normal garden soil. The percentage of survival was recorded 15 days after transfer of acclimatized plantlet to greenhouse.

6.2.4 *Agrobacterium* strain, binary plasmid and bacterial culture

The binary vector pCAMBIA2301 was introduced to *A. tumefaciens* strain EHA105 and used for transformation studies (Fig. 6.1). The T-DNA of pCAMBIA2301 includes neomycin phosphotransferase gene (*nptII*) and β-glucuronidase (*gus*) interrupted by catalase intron, both driven by the cauliflower mosaic virus (CaMV) 35S promoter.

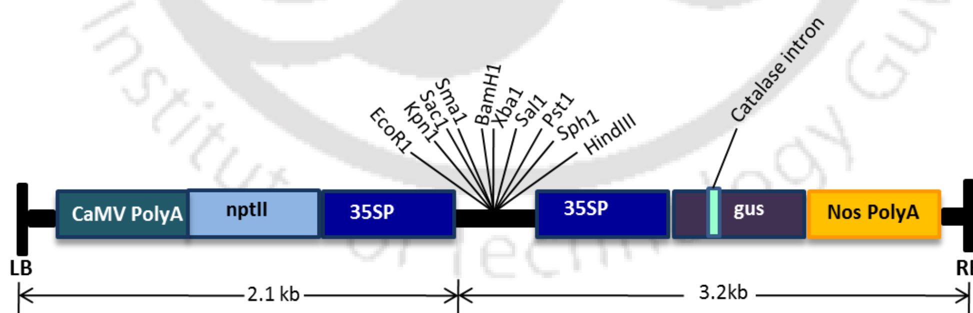

Fig. 6.1 **T-DNA region of pCAMBIA 2301 plasmid**. RB, Right border; CaMV 35S Promoter or terminator; NOS, Nopaline synthase terminator; Intron gus, intron interrupted beta-glucuronidase: *npt* II, Neomycin phosphotransferase

A single colony of the bacterial strain was inoculated in 25 ml of liquid AB minimal medium (Chilton et al. 1974) containing 10 mg/L rifampicin and 50 mg/L kanamycin and grown overnight at 28 °C until O.D. of culture reached to 0.8. For infection, the cells

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were collected by centrifuging at 5,000 rpm for 5 min and the pellet was resuspended in liquid cocultivation medium, LCM (MS medium containing 6.66 µM of BAP and 0.24 µM of IBA) containing acetosyringone.

6.2.5 Transformation procedure and histochemical GUS-assay

The cotyledonary leaf segment explants of different age (obtained from freshly germinated seeds or 1–2 weeks old seedlings developed from embryos) were inoculated in bacterial suspension for 30 min with occasional shaking in dark. The explants were then blotted on sterile filter paper and co-cultivated in petridishes lined with filter paper, moistened with LCM supplemented with acetosyringone. After cocultivation, the explants were washed three to four times with sterile distilled water and blotted dry on sterile filter paper. Histochemical GUS assays (Jefferson et al 1987) were used to assess transient expression of the *gus* gene. Transient *gus* expression in leaf explants was scored after 3-day-cocultivation and the number of explants showing transient *gus* expression at their edges were scored by immersing the tissue materials in GUS substrate solution consisting of 0.5 M NaPO4 (pH 7.0), 50 mM potassium ferricyanide, 50 mM potassium ferrocyanide, 10 mM EDTA (pH 7.0), 0.1 % Triton X-100 with 1 g dm^{-3} of 5-bromo-4-chloro-3-indolyl-β-d-glucuronide (X-gluc) (Biosynth AG, Staad, Switzerland) for 24 h at 37°C. Following incubation, tissues were bleached with 100% ethanol and examined under microscope.

6.2.6 Optimization of factors affecting *Jatropha* transformation

The critical parameters influencing transformation efficiency were evaluated. Each experiment included three replicates of 20 explants each. All experiments were repeated three times. In order to evaluate the age of the explants on *Agrobacterium*-mediated transformation, cotyledonary leaf segment explants excised from freshly germinated seeds or 1–2 weeks old seedlings developed from embryos were used for transformation. Other parameters included the pH of co-cultivation medium (5.2, 5.4,

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5.7 or 5.9), acetosyringone concentration (0, 50, 100 or 150 µM), length of co-cultivation period (2, 3 or 4 days) and co-cultivation temperature (20, 22, 24, 25, 26 or 28 °C). Histochemical GUS assay was used to assess efficacy of different parameters on T-DNA transfer.

6.2.7 Regeneration of transformants and molecular analysis

Following co-cultivation, the explants were transferred to callus induction medium (MS salts, 3% sucrose, 6.66 µM of BAP and 0.24 µM of IBA) containing augmentin (400 mg/L) and cefotaxime (500 mg/L). After 3 weeks of culture, the calli were transferred to multiple shoot induction medium (MS salts, 3% sucrose, 6.66 µM BAP, 0.24 µM IBA and 1.44 µM GA_3) with augmentin (400 mg/L), cefotaxime (500 mg/L) and kanamycin (15 mg/L). Shoot survived on kanamycin selection medium were subcultured at two weeks interval.

Genomic DNA was isolated from the young leaves of putative transformed and non-transformed (control) *Jatropha* plants using the modified CTAB method (Khanuja et al. 1999) and were analyzed for the presence of the nptII and gus transgene by PCR amplification. The primers were designed to give amplification products of the internal sequence of 540 bp of the *nptII* and 240 bp of *gus*. The plasmid pCAMBIA2301 was used as positive control. The *nptII* and *gus* were amplified using respective 20 mers (nptII Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGCTA) and 24 mers (*gus* Fw: TAACCTTCACCCGGTTGCCAGAGG; Rv: CCTTAACTAAGCCGGAATCCATCG) oligonucleotide primers. The amplification reaction was carried out under the following conditions: 94°C for 5 min (1 cycle), 94°C for 1 min (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (extension) for 35 cycles followed by the final extension at 72°C for 7 min (1 cycle). PCR amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining under UV light (Sambrook et al. 1989).

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All parameters were evaluated and optimized on the basis of surviving shoots on kanamycin selection medium that showed positive amplification by polymerase chain reaction (PCR). All data were analyzed using ANOVA for a completely randomized design. Statistical differences among the means were analyzed by Newman-keul's multiple range test using the SPSS ver 7.5 (Snedecor and Cochran 1989).

6.2.8 Establishment of transformed plants

The green shoots developed on kanamycin selection medium were transferred to shoot elongation medium (SE, MS medium and 1.0 µM GA_3) with augmentin (400 mg/L), cefotaxime (500 mg/L) and kanamycin (15 mg/L). After 1 week the elongated shoots were transferred to root induction medium (RI, ½ MS medium supplemented with 0.5µM IBA and 100 mg/L augmentin). Plantlets with well-developed roots were acclimatized and maintained in the greenhouse as per the procedure described earlier in this study.

6.2.9 Statistical analysis

All the experiments of callus induction, shoot proliferation, and root formation were set up in a completely randomized design and each treatment had three replicates of 20 explants each. All data have been statistically analyzed by one-way ANOVA followed by Newman–Keul's multiple range tests for mean comparison (P=0.05). All statistical analyses were performed using the SPSS Statistics (version 19).

6.3 Results and discussions

6.3.1 *De novo* plant regeneration from cotyledonary leaf segments

Cotyledonary leaf segment explants (Fig. 6.2a) placed on the MS medium supplemented with 6.66 µM of BAP and 0.24 µM of IBA exhibited distinct morphological changes. The explants turned to green in color within a week of culture, enlarged and swelled at their edges within the following two weeks of incubation showing the sign of dedifferentiation (Fig. 6.2b). The explants formed white friable callus after 3 weeks of

culture. De novo shoot bud induction appeared at the edges of calli within two weeks of transfer of calli to MS medium containing 6.66 µM BAP, 0.24 µM IBA and 1.44 µM GA_3 at 16 h photoperiod regime (Fig. 6.2c and 6.2d). The cut edges of the leaves provided a way for nutrients and growth regulators to be absorbed efficiently from the medium (Sarwar and Skirvin 1997) and the synergistic effect of BAP and IBA triggering callus induction response was in agreement with previous reports in *Jatropha* (Sujatha and Mukta 1996; Weida et al. 2003; Li et al. 2007). Prior incubation in the dark was found very critical for dedifferentiation and redifferentiation of *Jatropha* in our studies. Prior incubation in the dark has been reported to increase shoot regeneration in various plant species, such as Zhanhua winter jujube (Gu and Zhang 2005), campanula (Sriskandarajah and Serek 2004), watermelon (Compton, 1999), quince (Baker and Bhatia 1993), pear (Chevreau et al. 1989), apple (Fasolo et al. 1989) and blueberry (Billings et al. 1988). Incubation in the dark has been reported to delay degradation of endogenous and/or exogenous plant growth regulators (Rusli and Pierre 2001) besides reducing the levels of cell wall thickness and cell wall deposits (cellulose and hemicellulose), facilitating translocation of plant growth regulators in plant cells (Herman and Hess 1963). The calli developed cluster of multiple shoots (Fig. 6.2e) with an average of 12.56 shoots in 94% of the responded calli (Table 6.2) within 4 weeks of transfer of callus to shoot induction medium. Cotyledonary leaf explants have been most widely studied for *de novo* shoot formation in diverse plant species (Nikam and Shitole 1997; Amutha et al. 2003; Vengadesan et al. 2003).

6.3.2 Influence of age and orientation of explants on callus induction and shoot multiplication

The factors that significantly influenced the efficiency of callus induction and prolific de novo plant regeneration in cotyledonary leaf segment explants of *Jatropha* were the age of the explant and its orientation in culture.

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Table 6.1 Effect of age of explant on callus formation and multiple shoot induction from cotyledonary leaf segment explants of *J. curcas*

Age of the cotyledonary leaf explant (days)	Response of the cotyledonary leaf explant		
	Callus formation (%)	**Average no of shoots**	**Regeneration frequency (%)**
0	$87.50^{a} \pm 3.46$	$12.56^{a} \pm 0.38$	$87.50^{a} \pm 3.11$
7	$75.83^{ab} \pm 3.46$	$4.35^{b} \pm 0.32$	$65.83^{b} \pm 3.27$
14	$70.83^{bc} \pm 3.46$	$2.66^{c} \pm 0.35$	$57.50^{bc} \pm 3.24$

Although the explants of different age formed callus and subsequently induced clusters of multiple shoots, the percentage of explants that formed callus, the percentage of callus that induced multiple shoots and the average number of shoots per callus significantly declined with the increase in age of the explants (Table 6.1). The best regeneration response was observed in explants, prepared from cotyledonary leaves of freshly germinated seeds with 87.5% of the explants forming callus, out of which 94% of the callus regenerated forming an average of 12.56 shoots, a response which was significantly higher than one- and two-week-old explants (Table 6.1). Famiani et al (1994) proposed that explants from young leaves show more regeneration potential than older leaves as the younger leaves, still developing, have less differentiated and more metabolically active cells. Therefore, under suitable hormonal and nutritional conditions, show improved plant regeneration. Furthermore, explants of different age may have different levels of endogenous hormones. Therefore, the age of explants would have a critical impact on the regeneration efficiency. Similar results have been reported in other plants, including *Platanus occidentalis* (Sun et al. 2009), *Morus alba* (Thomas, 2003), *Cajanus cajan* (Dayal et al. 2003), *Rosa hybrida* (Ibrahim and Debergh 2001), *Aerides maculosum* (Murthy and Pyati 2001), *Cercis canadensis* (Distabanjong and Geneve 1997), Malus (Famiani et al. 1994), *Cydonia oblonga* (Baker and Bhatia 1993),

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Aegle marmelos (Islam et al. 1993), Lachenalia (Niederwieser and Van Staden 1990) and Prunus (Mante et al. 1989).

Fig. 6.2 (a–l) **De novo shoot bud induction and plant regeneration from cotyledonary leaf explant of *J. curcas*** (a) Cotyledonary leaf segment explant of freshly germinated seed (bar: 0.7 cm) (b) White, friable callus induced from cotyledonary leaf segment on MS medium with BAP (6.66 µM) and 0.24 µM indole-3-butyric acid (IBA) after 3 weeks of dark incubation (bar: 1.4 cm) (c) De novo shoot bud induction from cotyledonary leaf segment derived callus on MS medium supplemented with BAP (6.6 µM), IBA (0.24 µM) and GA_3 (1.44 µM) after 2 weeks of culture at 16 h photoperiod regime (bar: 1.8 cm) (d) Microscopic view of leaf edge showing shoot bud induction (bar: 0.15 mm) (e, f) Profuse shoot bud clump formation from cotyledonary leaf segment derived callus (g) Shoot elongation on MS medium supplemented with GA_3 (1.0 µM) after 2 weeks of culture (h) Rooting of *in vitro* regenerated shoot on half strength MS medium supplemented with NAA (5.00 µM) within 17 days of culture (i) An acclimatized plant (j) Plants established in nursery (k, l) GUS expression at the cut edges of cotyledonary leaf segment immediately after cocultivation with *Agrobacterium tumefaciens* EHA105pCAMBIA2301 (k) and untransformed control leaf explant (l)

In contrast with the above results, a higher regeneration response was observed from explants of older leaves as compared to the younger explants in apple (Antonelli and

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Druart 1990). Success of regeneration from leaf explants depends not only on the age of the explant chosen, but also the way explants are placed on the culture media (Duzyaman et al. 1994; Bhatia et al. 2005). Explants can be inoculated on the culture media in abaxial (lower surface facing down) or adaxial (upper surface facing down) orientation for the cotyledons and leaves (George, 1993). The best results were obtained when the leaf abaxial side was touching the culture medium as in this position the regeneration obtained was nearly two-fold higher than the leaf explants cultured with their adaxial side in contact with the medium (Table 6.2). Similar observations have been reported with the leaf abaxial side touching the culture medium (Dolcet-Sanjuan et al. 1990; Fiola et al. 1990; Leblay et al. 1990; Stamp et al. 1990; Welander and Maheswaran, 1992; Duzyaman et al. 1994; Bartish and Korkhovoi, 1997).

Table 6.2 Effect of orientation of explant in the culture medium on callus formation and multiple shoot induction from cotyledonary leaf segment explants of *J. curcas*

Orientation of the cotyledonary leaf disc attached to the medium	Response of the leaf disc		
	Percentage of callus formation	Regeneration frequency (%)	Average no of multiple shoots
Adaxial	87.84[a] ± 4.32	87.62[a] ± 4.06	12.54[a] ± 0 .38
Adaxial	54.23[b] ± 3.63	56.34[b] ± 4.45	07.35[b] ± 0 .42

Shoots produced from the adaxial orientation protruded into the culture medium and then turned upwards, trying to emerge from the medium. This resulted in a reduced number of shoots being produced per explant. The contrasting response of the explants in its two different orientations is attributed to the total surface area of the explants that come in contact with the medium.

6.3.3 Elongation of shoots

In the present study, shoot clumps produced on caulogenesis medium, either took prolonged duration for elongation of a few shoot buds or majority of the shoots buds

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turned necrotic and later died on being transferred to phytohormone-free basal medium, a response which is quite prevalent in plant regeneration from callus (Ibrahim and Debergh 2001). It appears that the hormone levels present in the medium used for shoot induction have a carry-over effect in the shoots. This situation caused prolific multiplication of shoot initials from the calli and this process prevented individual shoot elongation. It was necessary to develop a suitable medium for faster elongation of shoot buds. Incorporation of 1.0 µM GA_3 to MS media significantly enhanced the shoot elongation within one to two weeks of culture (Table 6.3; Fig. 6.2g), as compared to medium devoid of GA_3.

Table 6.3 Effect of gibberellic acid (GA_3) on elongation of shoots from cotyledonary leaf segment derived callus of *J. curcas* on MS medium after two week of culture

GA_3 (µM)	Mean shoot length (cm)	Fold increase in shoot length
0	$1.37^{a} \pm 0.07$	0
0.5	$2.90^{b} \pm 0.03$	$1.11^{b} \pm 0.03$
1.0	$5.12^{c} \pm 0.03$	$2.73^{a} \pm 0.03$
1.5	$3.22^{d} \pm 0.03$	$1.35^{b} \pm 0.03$
2.0	$3.35^{e} \pm 0.03$	$1.44^{b} \pm 0.03$

The promotive effect of GA_3 on elongation of stunted shoots has been reported in several other plant species (Demeke and Hughes 1990; Jordan and Oyanedel 1992; Purohit and Singhvi 1998; Sugla et al. 2007; Purkayastha et al. 2008). GA_3 is considered to stimulate shoot elongation by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger 1998).

6.3.4 Rooting and transplantation

Rhizogenesis is the final step in the formation of complete plantlet in *in vitro* culture. Root formation occurred in 40% of the shoots within 20–22 days of culture on half strength MS medium. No roots could be induced on full strength MS basal medium, wherein the shoot bases turned brown indicating necrosis effect. Roots were not induced on medium containing IBA and the shoots formed varying degree of callus at

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shoot base (Table 6.4). However, NAA containing medium induced roots without any callus induction at the shoot base (Table 6.4). Highest percentage of root induction occurred in 75% of the shoots in the medium containing 5.0 µM NAA within 17–20 days of culture (Fig. 6.2h).

Table 6.4 Effect of different concentrations of auxins (IBA and NAA) added to half strength MS medium on rooting of shoots raised from cotyledonary leaf derived callus of *J. curcas*

Auxin (µM)	Percentage of rooting	Degree of callus formation at shoot base	No of days required for rooting
	40^{a} ± 3.71	-	20-22
IBA			
1	-	+	
2.5	-	+ +	
5	-	+ + + +	
NAA			
1	-	-	-
2.5	55^{b} ± 4.26	-	19- 22
5	75^{c} ± 4.39	-	17- 20

The contrasting difference between the present observation and our previous report (Purkayastha et al. 2010), wherein all the shoot apex-derived shoots induced roots in half strength medium could be attributed to the origin of shoots. Plantlets with well-established roots were successfully acclimatized (Fig. 6.2i) in pots containing soil, vermiculite and vermicompost (1:1:1) and eventually established in a nursery (Fig. 6.2j) with survival frequency of 97%. The established plants were apparently uniform and did not show any observable variation.

6.3.5 Optimization of parameters influencing *Agrobacterium*-mediated transformation

6.3.5.1 Influence of age of explants

Agrobacterium-mediated transformation has often proved unsuccessful due to the accumulation of secondary metabolites particularly complex mixtures such as the oils (Sugimura et al. 2005) or bacteriostatic polyphenols (Kumar et al. 2004) in the explants of many plants. Therefore, selection of leaves of appropriate developmental stage that

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possess low amounts of oil, without compromising the prolific regeneration ability of the explants, is crucial for establishing successful *Agrobacterium* infection and transgenic plant generation. In order to select leaf materials for their greater amenability to *Agrobacterium*-mediated genetic transformation, the transient *gus* expression analysis was performed on explants from cotyledonary leaves of different age, following three days of cocultivation with *A. tumefaciens*. The cotyledonary leaf segment explants excised from one- and two-week-old *in vitro* raised seedlings were compared with those excised from freshly germinated seeds for their transformation efficiency. Strong *gus* expression was observed at the edges of younger explants (Fig. 6.2k) as compared to older explants. The endogenous *gus* activity (color) was not detected in non-transformed (control) explants (Fig. 6.2l). The *gus* activity at the cut ends indicated the amenability of explants to *Agrobacterium*-mediated transformation. A significant difference in percentage of explants showing *gus* expression was observed among the explants of different ages (Fig. 6.3a). The number of *gus* expressing explants was significantly higher in the case of cotyledonary leaf explants, excised from the freshly germinated seeds, as compared to the explants excised from one-and two-week-old leaves (Fig. 6.3 a).

6.3.5.2 Effect of co-cultivation medium pH

Several research groups have reported that optimal induction of vir genes is attained when the pH of co-cultivation medium is lower than those of commonly used plant tissue culture medium (Stachel et al.1985; Vernade et al. 1988). An acidic pH of co-cultivation medium is known to induce optimal expression of virulence genes of *Agrobacterium* (Stachel et al. 1985). Therefore, the effect of acidic pH during co-cultivation of cotyledonary leaf segment explants was tested in the range of 5.2–5.8. A co-cultivation medium pH of 5.7 was found optimal for T-DNA delivery to explants as indicated from transient *gus* expression frequency (Fig. 6.3b) and a significant decrease

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in transient *gus* expression frequency was observed at co-cultivation medium pH of 5.9 (Fig. 6.3b).

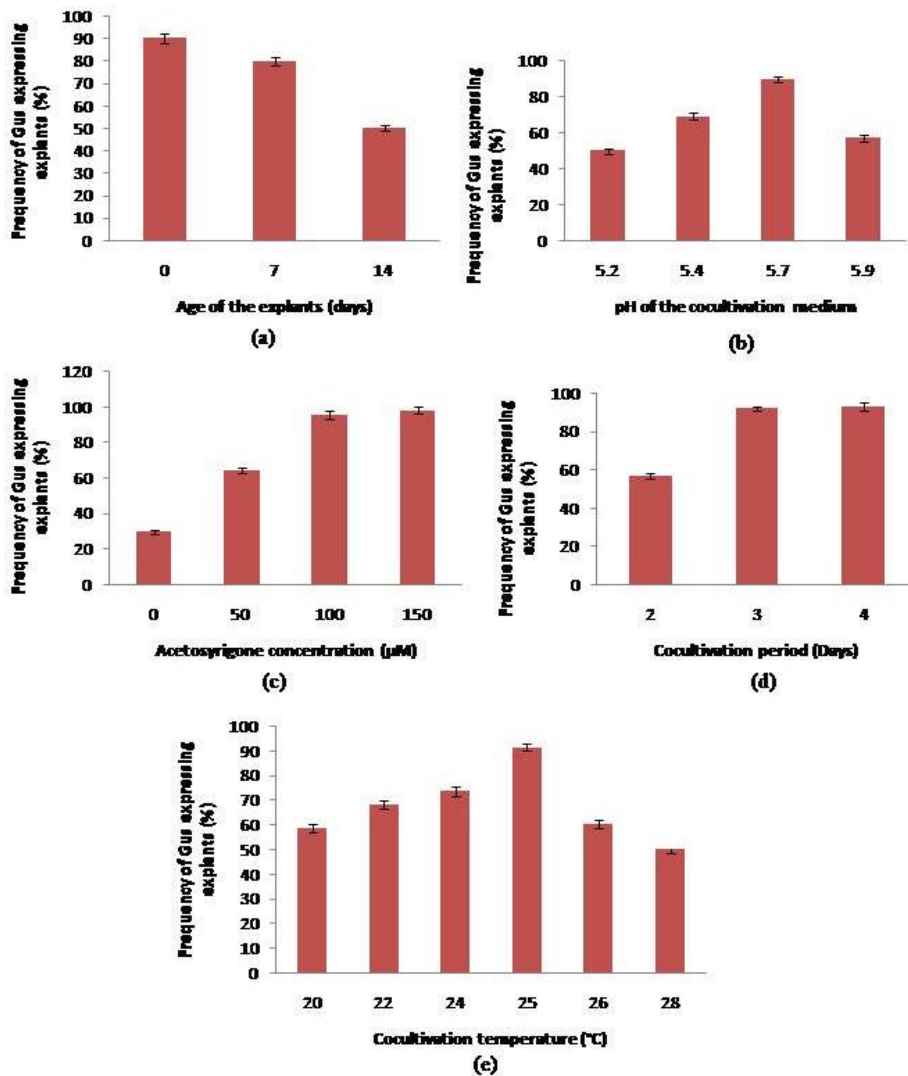

Fig. 6.3 (a-e) **Optimization of various factors affecting the efficient T-DNA delivery to cotyledonary leaf explants of *J. curcas* in *Agrobacterium*-mediated transformation** (a) Effect of age of the cotyledonary leaf explants on transient gus expression (b) Effects of pH (5.2–5.9) of the cocultivation medium on gus expression (c) Effects of the various concentrations (0, 50, 100, 150 µM) of acetosyringone during cocultivation (d) Effect of cocultivation time (2, 3, 4 day) on gus expression of cotyledonary leaf explants (e) Effect of cocultivation temperature on frequency of gus expression of cotyledonary leaf explants. Values represent the mean±SE of five independent cocultivation experiments (P=0.05).

The effect of the pH of the co-cultivation medium on transformation efficiency has been reported in several plant species (Aliev et al. 1988; Meurer et al. 2001; Shrivastava et al. 2001; Kumar, 2003; Paz et al. 2006; Shrawat et al. 2007; Bartlett et al. 2008). Kumar et

al (2010f) reported acidic pH in co-cultivation medium most effective for transforming leaf explants of 4 weeks-old *in vitro* grown *Jatropha.*

6.3.5.3 Effect of aetosyringone concentration

Phenolic compound, acetosyringone secreted by wounded tissues is known to induce *Agrobacterium* vir genes (Stachel et al. 1985) and thereby enhance *Agrobacterium*-mediated transformation (Ozawa, 2009; He et al. 2010). In order to determine the appropriate concentration of acetosyringone for optimal T-DNA delivery, four different concentrations of acetosyringone were tested in inoculation media (Fig. 6.3c). The transient transformation efficiency was found to increase with increase in concentration of acetosyringone up to 100 μM, which decreased with further increase in concentration (Fig. 6.3c). Maximum transient *gus* expression efficiency was recorded at 100 μM suggesting that presence of acetosyringone during inoculation of explants to *Agrobacterium* significantly improved transient transformation in cotyledonary leaf explants excised from the freshly germinated seeds of *Jatropha.* Our result on positive role of acetosyringone on transient transformation efficiency is in agreement with previous observations recorded in 14-d old (Li et al. 2007) and 4 weeks-old (Kumar et al. 2010f) leaf explants of *in vitro* grown *Jatropha*.

6.3.5.4 Effect of co-cultivation period

The cocultivation period was known to influence transformation efficiency (Saini et al. 2007). Co-cultivation period ranging from 2–7 days have been found to be suitable for *Agrobacterium*-mediated transformation of crop plants (Shrawat et al. 2007: Ahsan et al. 2007). The optimum transient transformation frequency was recorded when the explants were co-cultivated with *Agrobacterium* for 3 days. While longer co-cultivation period (4 days) was observed not only to reduced transient transformation efficiency but also resulted in *Agrobacterium* overgrowth leading to its detrimental effect on regeneration potential of the co-cultivated explants. Therefore, 3-day co-cultivation was

adopted for transformation of cotyledonary leaf segments, excised from the freshly germinated seeds of *Jatropha* (Fig: 6.3d).

6.3.5.5 Effect of co-cultivation temperature

Cocultivation with *Agrobacterium* at lower temperature has been shown to improve T-DNA transfer in to several plant species (Dillen et al. 1995; Salas et al. 2001; Sunilkumar and Rathore 2001). Maximum transient *gus* transformation efficiency was recorded at co-cultivation temperature of 25°C, whereas decrease in transient transformation frequency was observed both below and above co-cultivation temperature of 25°C (Fig 6.3e). The results suggested that 25°C is most suitable cocultivation temperature for efficient T-DNA delivery to cotyledonary leaf segments, excised from the freshly germinated seeds of *Jatropha.*

6.3.5.6 Improvement of the transformation method and regeneration of transformed *Jatropha* plants

Regeneration of plants via *de novo* shoot organogenesis which relies on the capacity to regenerate entire plant from a single somatic cell (Duclercq et al. 2011) presents an attractive target for recovery of genuinely transformed plants through *Agrobacterium*-mediated transformation as it minimizes the risks of generation of escapes and chimeras (Pniewski and Kapusta 2005). In our study, we adapted *de novo* organogenesis mode of regeneration from cotyledonary leaf explants for *Agrobacterium*-mediated transformation. We found younger explants more amenable to *Agrobacterium*-mediated transformation (Fig. 6.3a). The cotyledonary leaf segment explants excised from the freshly germinated seeds were most amenable to T-DNA transfer and transformed explants induced calli on kanamycin free callus induction medium as callus induction from cotyledonary leaf segment explants was inhibited in presence of kanamycin (data not shown). Kanamycin resistant shoots were successfully recovered within 2-3 weeks of culture on the multiple shoot induction medium containing 15 mg/L kanamycin (Fig. 6.4a and b).

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Fig. 6.4 (a–k) Stages of development of transgenic plants by *Agrobacterium tumifaciens* mediated transformation of *J. curcas* harboring pCAMBIA2301 (a-b) Selection of regenerated shoot on kanamycin containing medium (bar: 1.2 cm) (c) Elongation of kanamycin resistant shoot (bar: 1 cm) (d) Rooted transformed plant (bar: 1 cm) (e) Acclimatized transformed plants (f) Stable Gus expression of the flower of T_0 plant and control plant (g) (bar: 0.2 cm) (h) Stable Gus expression in fruit transverse section of T_0 plant (bar: 0.8 cm) and non-transformed plant (i) (bar: 0.8 cm) (j) Stable Gus expression in longitudinal section of seed of theT_0 plant (bar: 0.3 cm) and non-transformed plant (k) (bar: 0.3 cm)

Pan et al (2010) also indicated the callus induction process from cotyledonary leaves in *Jatropha* being sensitive to low concentration of kanamycin. The kanamycin resistant shoots elongates on transfer to MS medium containing 1.0 µM GA_3, 15 mg/L kanamycin and formed root in MS medium containing 0.5 µM IBA devoid of kanamycin within 2-3 weeks of culture (Fig. 6.4 c, d). *Agrobacterium*-mediated transformation of *Jatropha* using cotyledonary leaf explants was reported by Li et al (2007). However, this was inefficient and difficult to reproduce (Mishra and Mishra 2010). Kumar et al (2010f) reported generation of transgenic *Jatropha* from 4 weeks-old leaf explants of *in vitro* grown plants. Our results on high shoot regeneration competence of cotyledonary leaves of freshly germinated seeds are in agreement with a recent report of *Jatropha* transformation using leaf explants (Tsuchimoto et al. 2012). However, authors adapted

younger leaves instead of cotyledonary leaves for shoot *Agrobacterium*-infection and shoot multiplication on TDZ containing medium and encountered carry-over effect of TDZ on regenerated transformed shoots which posed difficulties in rooting (Tsuchimoto et al. 2012). This could be attributed to varietal effect on regeneration as the authors used Philippine, Thai, and Tanzanian lines for transformation (Tsuchimoto et al. 2012).

6.3.5.7 Stable GUS assay and molecular analysis

A strong, uniform and stable *gus* expression was detected in flower, fruit and seed of kanamycin resistant regenerated shoots (Fig.6.4 f, h, j) and no endogenous *gus* expression was detected in the tissues of control untransformed shoots (Fig.6.4 g, I, k). To confirm the presence of the transgenes in the putatively transformed plants, GUS-positive kanamycin resistant regenerated plants and non-transformed plants were subjected to PCR analysis. The presence of the *npt*II and *gus* genes in kanamycin resistant and *gus* positive plants was confirmed by detection of the expected 240 bp and 580 bp amplification products, respectively (Fig.6.5 a, b). No amplification product was detected in DNA from control (non-transformed) shoots.

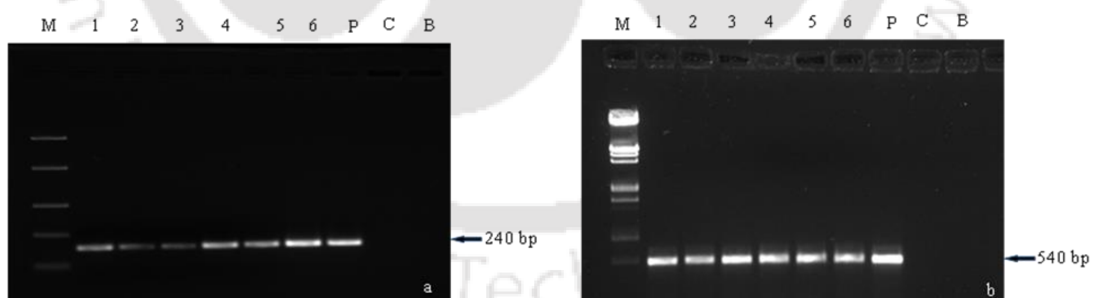

Fig. 6.5 (a-b) Molecular analysis of transgenic plants (a) PCR amplification of the 240 bp fragment *of Gus* gene (b) PCR amplification of the 540 bp fragment of *nptII* gene. Lane M: molecular weight marker; Lane 1, 2, 3, 4, 5, 6: genomic DNA from six transgenic plant; Lane P: pCAMBIA2301 plasmid (positive control); Lane C: DNA from transformed plant (negative control); Lane B: blank

6.4. Conclusion

In the present study, a simple and efficient *Agrobacterium* mediated genetic transformation system for *J. curcas* using cotyledonary leaf explants was established. The choice of explants of appropriate age, and the orientation of the explants in culture

medium were found to exert significant influence on the frequency of *de novo* plant regeneration. Furthermore, the age of the explant was found to be the critical aspect in conferring appropriate biological condition of the explant vital for optimal infection and T-DNA transfer by *Agrobacterium tumefaciens*. Highest regeneration response was reported in the young explants, derived from the cotyledonary leaves of germinating seed compared to the leaves from one- and two-week-old seedlings. This gradient with age of the explant was observed in frequency of callus induction and shoot organogenesis from callus. The explants cultured with their abaxial side in medium showed significantly higher regeneration response. The youngest explant was found to be most amenable to *Agrobacterium* mediated transformation as compared to older explants giving rise to stable transgenic plants in *J. curcas*. This efficient transformation protocol may facilitate the genetic manipulation of *J. curcas* for desired traits and can lead to a better understanding and improvement of the biofuel species.

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Chapter 7

Generation and evaluation of transgenic *Jatropha* expressing *cry1Ac* gene

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7.1 Introduction

Jatropha curcas is an important non-edible oilseed crop receiving worldwide attention as a biodiesel feedstock. The seeds which contain 30-42% of oil can be directly blended with diesel or transesterified for use as biodiesel (Agarwal et al. 2007). The short gestation period, drought endurance, low cost of seeds, high oil content, and easy adaptation on marginal and semi marginal lands make *Jatropha* as the most sought oil seed crop among the non-edible oil yielding crops for biodiesel production (Tatikonda et al. 2009). The huge demand of *Jatropha* seeds is expected to be met out through large scale cultivation of *Jatropha* and increasing its productivity. However, large scale cultivation of *Jatropha* through monocrop plantation especially under humid conditions encounters with devastation by flower, inflorescence and capsule feeding insects *Scutellera nobilis* and *Pempelia morosalis* in India (Akbar et al. 2009) and *Pachycoros klugii* Burmeister in Nicaragua (Shanker and Dhyani 2006), affecting flower fall, fruit abortion and malformation of seeds causing huge economic losses. The larvae of the polyphagous tobacco cut worm, (cluster caterpillar) *Spodoptera litura* feed on *J. curcas* leaves and this moth occurs over the northern two-thirds of Australia (Herbison-Evans and Crossley 2006). Seed productivity is drastically reduced due to attack of lepidopteron insect, *S. litura*, which causes massive defoliation in monoculture. The damage due to *S. litura* insect was estimated to be in the range of 60 % -70% in *Jatropha* (Meshram and Joshi 1994). Control of insects through spraying insecticides is known to compromise with emergence of resistance biotypes and posing severe problem to environment and ecosystem. Large scale cultivation of *Jatropha* therefore, requires the development of insect resistant varieties.

The scope of classical breeding is limited due to narrow genetic base and longer breeding cycle (Natarajan et al. 2010). Consequently, the transfer of insect pest resistance genes by genetic transformation could potentially aid plant breeders in

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overcoming the production constraints and accelerate the development of resistant cultivars for breeding programs. Introduction and expression of insecticidal *cry* genes of *Bacillus thuringiensis* (Bt) through transgenic technology has been reported to develop durable resistance to target insects in several oil seed crops including canola (Stewart et al. 1996), oil palm (Lee et al. 2006) and castor (Kumar et al. 2011). Overexpression of a hybrid *cry* gene based on *cry1Ac* has shown to impart resistance to *Spodoptera litura* and *Helicoverpa armigera* in the transgenic groundnut (Entoori et al. 2008). The incorporation and expression of *cry1Ac* in *Jatropha* is expected increase in mortality and possibly delay in development of *S. litura* enabling reduction of leaf defoliation and seed productivity.

In order to develop insect resistant variety of *Jatropha*, we introduced *cry1Ac* through an improved *Agrobacterium*-mediated transformation in *Jatropha* using juvenile cotyledonary leaf segment explants. Stable transgenic *Jatropha* plants expressing *cry1Ac* were recovered by kanamycin selection which showed presence, integration, expression and inheritance of transgenes.

7.2 Materials and methods

7.2.1 Plant material and explant preparation

Seeds were obtained from the *J. curcas* elite clones (IITJ20), which were preselected on our previous studies (Mazumdar et al. 2012). The seeds were decoated and soaked in distilled water overnight at room temperature. The soaked decoated seeds were treated with a 0.1% sodium hypochlorite solution containing few drops of Tween-20, for 10 min followed by washing under tap water for 20 min. The decoated seeds were then surface sterilized with 70% alcohol for 5 min followed by with 0.2% mercuric chloride for 10 min, and finally rinsed four times with sterile double distilled water. After blot-dried on sterile filter paper, the endosperm was carefully dissected out to expose embryos with papery cotyledonary leaves. The papery cotyledonary leaves were separated out and cut

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into four segments (10 mm^2) with the edges removed and used as explants for *Agrobacterium*-mediated transformation experiments.

7.2.2 Binary plasmid, *Agrobacterium* strain and culture conditions

The binary plasmid pCAMBIA230135S::*cry1Ac* containing a *cry1Ac* gene driven by CaMV2X35S promoter (Fig. 7.1) was mobilized into the disarmed hypervirulent *Agrobacterium tumefaciens* strain EHA105 and used for transformation experiments. The T-DNA of pCAMBIA2301 includes neomycin phosphotransferase gene (*npt*II) and β-glucuronidase gene (*gus*) interrupted by catalase intron, both driven by the cauliflower mosaic virus (CaMV) 35S promoter. The *A. tumefaciens* strain harboring pCAMBIA230135S::*cry1Ac* was maintained on solid YEP medium (An et al. 1988) supplemented with 10 mg/L of rifampicin, and 50 mg/L of kanamycin. Single bacterial colony was inoculated into 25 ml of liquid AB minimal medium (Chilton et al. 1974) with appropriate antibiotics and grown overnight at 28°C on a rotary shaker at 180 rpm, until optical density at 600 nm reached to 0.8. The cells were collected by centrifuging at 5,000 rpm for 5 min, and then the pellet was resuspended in liquid co-cultivation medium, LCM (MS medium containing 6.66 µM of BAP and 0.24 µM of IBA, pH adjusted to 5.7) supplemented with 100 µM acetosyringone and used for inoculation.

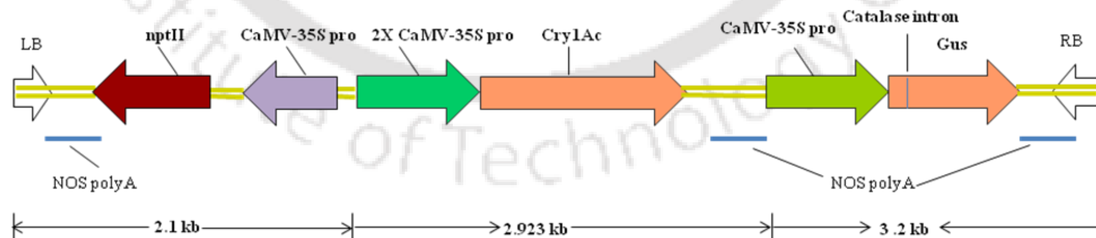

Fig. 7.1 T-DNA region (8.2 kb) of pCAMBIA23012X35S::*Cry1Ac* plasmid. RB, Right border; CaMV 35S Promoter or terminator; NOS, Nopaline synthase terminator; Intron gus, intron interrupted beta-glucuronidase: *npt* II, Neomycin phosphotransferase

7.2.3 Plant transformation and recovery of transformants

Transformation of *Jatropha* and generation of the primary transformants were accomplished by an improved transformation procedure as standardized earlier and described in the previous chapter. The juvenile cotyledonary leaf segment explants were inoculated in bacterial suspension at 25°C for 30 min by occasional shaking in dark. The explants were then blotted on sterile filter paper and co-cultivated for 3 days under dark condition at 25°C, in petridishes lined with filter paper moistened with LCM supplemented with 100 µM acetosyringone. After 3 days of co-cultivation, the explants were washed with 0.1% Tween 20 and five to six times with sterile distilled water followed by rinsing with sterile distilled water containing 500 mg/L cefotaxime. The explants were then blotted dry on sterile filter paper and transferred to callus induction medium (CI, MS medium supplemented with 6.66 µM BAP and 0.24 µM IBA) containing 500 mg/L cefotaxime and 400 mg/L augmentin in dark condition for callus induction. The cultures were transferred to fresh callus induction medium at an interval of 5, 7 and 8 days. After 3 weeks of culture, the calli were transferred onto shoot regeneration medium (SR, MS medium supplemented with 6.66 µM BAP, 0.24 µM IBA, 1.44 µM GA_3) containing 15 mg/L kanamycin, 500 mg/L cefotaxime and 400 mg/L augmentin, and incubated at 16 h photoperiod. Both regeneration controls (no cocultivation, no selection) and transformation controls (no cocultivation but selection on kanamycin) were used for all the experiments and at all cycles of selection. The cultures were periodically transferred onto fresh selection medium. After 4 weeks of culture on selection, the proliferating kanamycin resistant shoots were detached and transferred to shoot elongation medium (SE, MS medium supplemented with 1.0 µM GA_3) containing 15 mg/L kanamycin and 400 mg/L augmentin. After a week, the elongated shoots were transferred to root induction medium (RI, ½ MS medium supplemented with 0.5µM IBA) and 400 mg/L augmentin. Well-rooted transformed plantlets were washed

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thoroughly in running tap water and acclimatized and maintained in greenhouse as per the procedure described earlier.

7.2.4. Molecular analysis of the putative transgenic plants

Molecular characterization of the transformants was carried out by PCR, Southern hybridization, GUS histochemical analysis of different plant tissues, RT-PCR and ELISA analysis for confirmation of the presence, integration, expression and inheritance of the introduced genes.

7.2.4.1 PCR analysis

Genomic DNA was isolated from the young leaves of putative transgenic and nontransgenic *Jatropha* plant using the modified CTAB method (Khanuja et al. 1999) and was analyzed for the presence of *cry1Ac* and *npt*II transgene(s) by PCR amplification. The 540 bp region of *npt*II and 1 kb coding region of *cry1Ac* were amplified using respective 20 mers (*npt*II Fw: CCACCATGATATTCGGCAAC; Rv: GTGGAGAGGCTATTCGGC TA) and 24 mers (*cry1Ac* Fw:CCCAGAAGTTGAAGTACTTGGTGG; Rv: CCGATATTGAAGGG TCTTCTGTAC) oligonucleotide primers. The amplification reaction was carried out under the following conditions: 94°C for 5 min (1 cycle), 94°C for 1 min (denaturation), 58°C for 1 min (annealing), 72°C for 1 min (extension) for 35 cycles followed by the final extension at 72°C for 7 min (1 cycle). PCR amplified products were resolved by electrophoresis on 1% agarose gel and visualized by ethidium bromide staining under UV light (Sambrook et al. 1989).

7.2.4.2 Southern hybridization

Southern hybridization was performed to analyze the integration of the *cry1Ac* in genome of three randomly chosen PCR positive T_0 transgenic plants. About 60 µg of genomic DNA was isolated from randomly selected transgenic and nontransgenic (negative control) *Jatropha* plants and were digested overnight at 37°C with HindIII (10 units/ µg of DNA). HindIII digested pCAMBIA2301*cry1Ac* used as positive control. All

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digested products are fractioned on 0.8% agarose gels and blotted onto a positively charged Zeta-Probe membrane (Bio-Rad, USA). The DNA was then cross-linked to the membrane by UV irradiation (1200 Joules in UVC 500, Amersham Biosciences) and hybridized to an non-radioactive DIG labeled DNA probe. The probe was produced by PCR-amplification of the 1 kb PCR product, corresponding to the coding region of *cry1A*c gene. The probe (20 ng) was labeled and Southern hybridization was performed using nonradioactive DIG labeling and detection system (Roche Diagnostics, Mannheim, Germany) following supplier's instructions.

7.2.4.3 Qualitative reverse transcription (RT)-PCR analysis

For RT-PCR analysis, total RNA was isolated from the PCR-positive transgenic T_0 plants using Ambion RNAqueous ™ RNA isolation kit from 100 mg of leaf tissue according to manufacturer's instructions. The integrity of RNA was verified by visualizing the RNA bands on 1.5% denaturing agarose gel (Sambrook et al. 1989). Five µg RNA was used as template for reverse transcription (RT) PCR. RT-PCR was performed by using First Strand cDNA Synthesis Kit (Fermentas, USA) according to the manufacturer's instructions. The house keeping gene actin was used as a control to indicate the amount of starting RNA. The sequences were designed from the *J. curcas* actin gene. The primers were 5'-CAAGTCATCACCATTGGAGCA-3' and 5'-GCCTCTTAATTTCGGCTTTAA-3' which gave a 590 bp product with cDNA. The PCR of the coding sequences of *cry1Ac* gene was carried out using gene specific primers as described earlier. The PCR products were electrophoresed on 1.0% agarose gels, detected by ethidium bromide staining and photographed through the gel documentation instrument (Bio-Rad, USA).

7.2.4.4 Quick Dip/Lateral Flow strip assay for detection Cry1Ac protein

To test for the presence of Cry1Ac protein, total soluble protein was isolated from leaves of T_0 transgenic lines using sample extraction buffer (DesiGen, India), according to the manufacturer's instructions. Quick detection of Cry1Ac protein in the leaves of T_0 plants

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was performed using the Immunodiagnostic XpresstripsTM (DesiGen, India) as per the manufacturer's instruction. The presence of the control band, which is highly intense (upper purple line) in the white result window, indicates the proper run of assay, whereas a test line (purple) just below the control band in the result window depicts the expression of Cry1Ac protein of the transgenic samples. Absence of test line signifies no expression of Cry1Ac.

7.2.4.5 Enzyme-linked immunosorbent assay (ELISA)

The plants found positive for expression of Cry1Ac protein in qualitative assay on Bt-express immunodetection strips, randomly selected for ELISA. ELISA was carried out using Desigen Quan T-ELISA-96 well plate kit (Desigen, Maharashtra, India) following suppliers' instruction. Total protein was extracted from 5 mg of lyophilized powder of terminal leaf of putative transgenic plants using 500 µl ice cold sample extraction buffer in a 1.5 ml eppendorf tube with the help of micro-pestle. For the estimation of Cry1Ac, the 96-well titre plate was coated with 150 µl per well (1:1,000) of goat anti-Cry1Ac antibodies. The plate was then loaded with 100 µl samples and buffer was used in control wells. The plate was incubated at 37^0 C for 1.5 h, followed by washing with wash buffer twice. After washing, the plate was incubated with alkaline phosphatase conjugated secondary antibodies at a dilution of 1:1,000 with 250 µl per well for 45 min at 37^0 C. The plate was then washed with wash buffer twice and 250 µl of freshly prepared substrate (p-nitro phenyl phosphate, 1 mg/ml) was added per well. The plate was incubated at room temperature in the dark for 30 min and reaction was stopped and readings recorded at 405 nm in a micro plate reader (Tecan, Switzerland).

7.2.5. Stable GUS assay

Stable *gus* expression was detected in various stages of cultures during regeneration (callus and *in vitro* shoot) and plant parts including leaf, flower and fruit of putative

transgenic plants following the histochemical procedure as described previously. The tissue from non-transformed cultures and plants served as control.

7.3 Results and discussions

7.3.1 Generation of *cry1Ac*-expressing plants

In our previous study of the effects of age and orientation of explants in culture for *de novo* plant regeneration, the leaf segment explants derived from the juvenile cotyledonary leaves and cultured with their abaxial side in contact with medium was found most efficient for plant regeneration as well as amenable to *Agrobacterium*-mediated transformation (Mazumdar et al. 2010). Therefore, juvenile leaf segment explants prepared from cotyledonary leaves of freshly germinated seeds were employed for *Agrobacterium*-mediated transformation of *Jatropha* with *cry1Ac* overexpression construct. Efficiency of an *Agrobacterium* mediated transformation depends on regeneration potential and amenability of the explants to infection. Previous studies have found cotyledons more susceptible to *Agrobacterium* infection than other explants such as petioles, hypocotyls, epicotyls, or leafs (Li et al. 2006). Moreover, cotyledons of *J. curcas* are easy to obtain from germinating seed and large enough to make many segments to be used as explants for transformation (Li et al. 2007). Leaf explants have been used successfully for *Agrobacterium*-mediated genetic transformation of several plant species (Petri et al. 2008; Xu et al. 2009).

We observed kanamycin (5 mg/L) was detrimental to callus induction from cotyledonary leaf explants. Therefore, calli were induced in kanamycin free medium (Fig. 7.2a). Kanamycin resistant shoots were successfully recovered within 4 weeks of culture on the multiple shoot induction medium containing a low concentration (15 mg/L) of kanamycin (Fig. 7.2b). Pan et al (2010) also indicated the callus induction process from cotyledonary leaves in *Jatropha* being sensitive to low concentration of kanamycin.

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Fig. 7.2 Transformation and regeneration of *J. curcas* using embryonic cotyledonary explants (a) formation of callus from cotyledonary leaf explants subjected to *Agrobacterium tumefaciens* mediated transformation and cultured on kanamycin-free CI medium (b) Selection of regenerated shoots from callus on SR medium supplemented with kanamycin (c) rooted transformed plant (d) transformed plant in pot (e) acclimatized transformed plant (f) GUS expression expressing callus developed after transformation (g) untransformed control callus (h) GUS-expressing *in vitro*-shoot (i) untransformed control shoot (j) GUS-expressing *in vitro* leaf explant (k) untransformed control leaf explant

However, we observed that a combination of 400 mg/L augmentin and 500 mg/L cefotaxime was sufficient to arrest *Agrobacterium* overgrowth during callus induction, shoot multiplication and plant regeneration without adversely affecting morphogenesis. Previous studies indicated the use of 500 mg/L cefotaxime (Li et al. 2007; Kumar et al. 2010), 200 mg/L cefotaxime (Khemkladngoen et al. 2011; Tsuchimoto et al. 2012), or 300 mg/L moxalactam sodium solution (Kajikawa et al. 2012) to arrest *Agrobacterium* overgrowth.

The kanamycin resistant shoots elongated within a week of culture on medium containing 1.0 µM GA_3 supplemented with 400 mg/L augmentin and 500 mg/L cefotaxime. Well elongated shoots (2-3 cm) induced root formation within 2 weeks of culture on ½ MS medium containing 0.5 µM IBA and 100 mg/L augmentin (Fig. 7.2 c).

Kanamycin was not added in root induction media as it inhibited root induction. The rooted plantlets were acclimatized with 95% success and maintained in the glasshouse (Fig. 2d, e). The morphological appearance and growth rate of transgenic plants were normal as compared to those of non-transformed plants generated *in vitro* and wild-type plants germinated from seeds. The plants resumed growth in greenhouse and reached maturity within two years.

7.3.2 Stable GUS expression

Transient GUS expression was detected in putative transformed callus (Fig. 7.2 f), regenerating kanamycin resistant shoot and *in vitro* leaf in culture (Fig. 7.2 h, j), wheras, no gus expression was observed in control explants (Fig. 7.2 i. k). A strong, uniform, and stable GUS expression was detected leaves, flower, anther and seed (Fig. 7.3 a, c, d, f, h, j) of putative kanamycin resistant plants established in greenhouse whereas, no blue staining was observed in tissue of untransformed plant (Fig. 7.3 b, e, g, i). Khemkladngoen et al (2011) reported successful recovery of transgenic *Jatropha* using juvenile cotyledonary leaf explants subjected to physical wounding (1 min of sonication) prior to *Agrobacterium* infection. The authors although reported an approximately 53% stable transformation rate but the estimation was based on GUS expression in explants. However, many kanamycin resistant shoots regenerated from transformed explants showed absence of GUS expression (Khemkladngoen et al. 2011) as opposed to results obtained in our study indicating detrimental effect of physical wounding on post co-cultivation regeneration of transformed cells on kanamycin selection. In all, 64 independent, putatively transformed shoots were selected out of 5,443 explants used in co-cultivation by two procedures of agro-infection.

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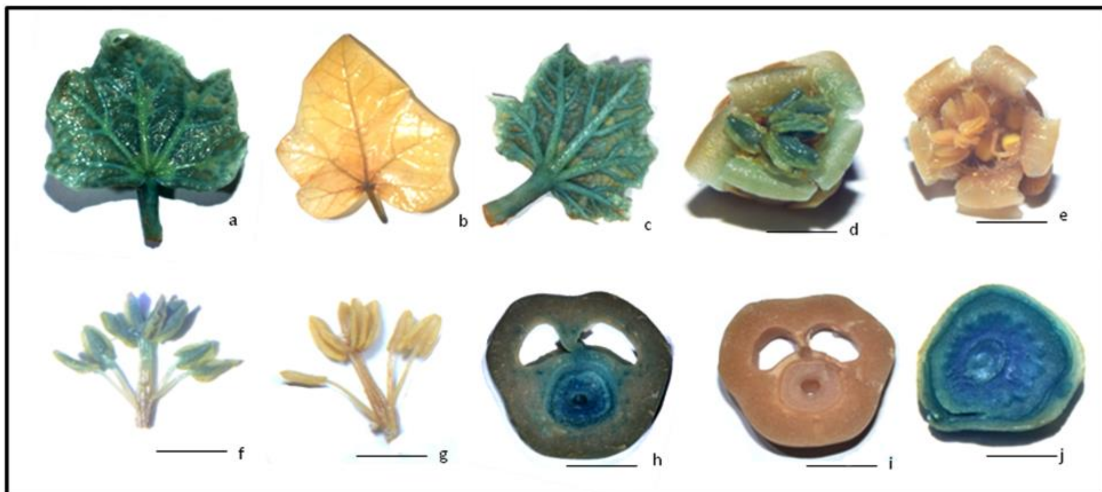

Fig. 7.3 Stable GUS expression in different part of leaf, flower and fruit of T_0 transgenic *Jatropha* plants (a, c) transgenic leaf (b) leaf of control plant (d) Stable GUS expression in male flower (bar = 5mm) (e) male flower of control plant (bar = 5mm) (f) anthers of transgenic plant (bar = 8 mm) (g) Anther of control plant (bar = 8mm) (h) transgenic fruit (bar = 1 cm) (i) control fruit (bar = 1 cm) (j) Transverse section of transgenic fruit (bar = 0.5mm)

Six putatively transformed shoots that finally survived at T_0 generation were subjected to rooting *in vitro*. The rooted plants were liquid-hardened and established in the clay pots containing sand and soilmixture (1:1) in glass house.

7.3.3 Molecular analysis of transgenic plants

A number of plants regenerated recovered on kanamycin selection medium were randomly subjected to PCR analysis to confirm the presence of transgenes and check the efficiency of kanamycin selection. The detection of the expected 540 bp and 1 kb amplified products corresponding to *npt*II and *cry1Ac* in PCR analysis confirmed the presence of the transgenes in T_0 transformed plants (Fig. 7.4 a, b). No amplification was detected in the control untransformed plants. The results clearly indicated the presence of transgenes in plants recovered on kanamycin selection medium demonstrating the efficiency of selection procedure established in our study.

The amplification of a 1 kb fragment corresponding to the *cry1Ac* in leaves of T_0 transgenic plants in RT-PCR analysis (Fig. 7.4 c) confirmed the accumulation of *cry1Ac* transcripts indicating the absence of gene silencing events.

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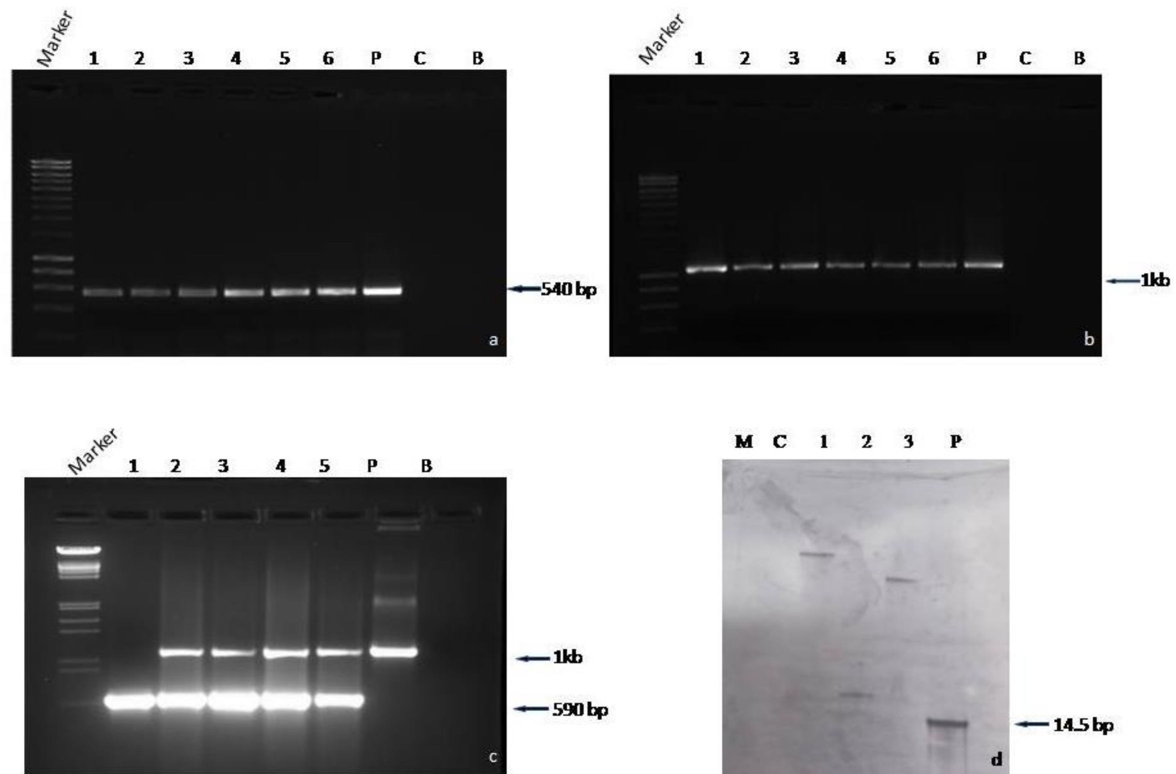

Fig. 7.4 Molecular analysis of transgenic *J. curcas* expressing *Btcry1Ac*. PCR amplification of the 540 –bp fragments of the *npt II* gene. Lane M: marker (Direct load wide range DNA marker, sigma) Lane 1, 2, 3, 4, 5, 6: genomic DNA from six transgenic plant; Lane p: pCAMBIA 2301 *Cry1Ac* plasmid (positive control); Lane C: DNA from untransformed plant (negative control): Lane B: Blank. (b) PCR amplification of the 1kb fragments of the *cry1Ac* gene. Lane M: marker (Direct load wide range DNA marker , sigma) Lane 1, 2, 3, 4, 5, 6: genomic DNA from six transgenic plant; Lane p: pCAMBIA2301*cry1Ac* plasmid (positive control); Lane C: DNA from untransformed plant (negative control): Lane B: Blank . (c) RT-PCR analysis of *Cry1Ac*, and reference gene *Actin*, Lane M λDNA/EcoR1+HindIII, Lane P: pCAMBIA2301*cry1Ac* plasmid DNA (positive control), Lane C: DNA from untransformed plant (negative control), Lane 1-5: transgenic *Jatropha* plants. (d) Southern blot hybridization analysis of junction fragments of three independently selected PCR positive T_0 lines. The plasmid and genomic DNA was digested with HindIII, and hybridized with *cry1Ac* probe, Lane 1-3: genomic DNA from three T_0 lines, lane C: genomic DNA from untransformed plant, lane P: HindIII digested pCAMBIA2301*cry1Ac*.

Furthermore, the amplification of the *cry1Ac* sequence from plant cDNA templates in RT-PCR ruled out the possibility of *Agrobacterium* contamination and efficiency of combination of 400 mg/L augmentin and 500 mg/L cefotaxime in eliminating *Agrobacterium* overgrowth from cultures.

Three randomly selected PCR-positive T_0 transgenic *Jatropha* plants were further screened by Southern analysis to confirm the integration of *cry1Ac* gene. Southern blot analysis of three T_0 transgenic plants are shown in Fig. 7.4 d. Hybridizations of DIG-labeled *cry1Ac* probe to total genomic DNA digested with *Hind*III

were expected to identify DNA fragments unique to individual integration events greater than 5.0 kb (Fig.7.4 d). All three randomly selected T_0 transgenic plants were found positive for *cry1Ac* and furthermore, they showed differential integration events, confirming that these plants were derived from independent transformation events (Fig. 7.5 d, lanes 1, 2, and 3). The T_0 transgenic plants exhibited simple hybridization patterns demonstrating single integration events (Fig. 7.4 d).

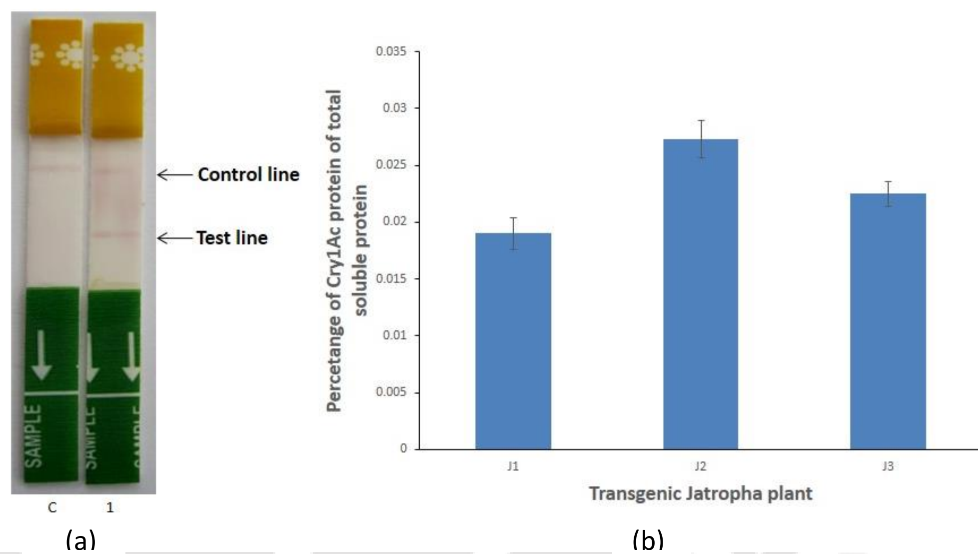

Fig. 7.5 Protein expression analysis of transgenic *J. curcas* expressing *Btcry1Ac* (a). Rapid immunodiagnostic test for detection of Cry1Ac in To transgenic lines using Desigen XpresstripsTM, C: no test line appeared from control plants after lateral flow assay; 1: transgenic line showed test line signifies the presence of Cry1Ac. (b) Expression level of Bt*Cry1Ac* protein in transgenic *Jatropha* lines (J1, J2, J3) from enzyme linked immunosorbant assay (ELISA). Values represent the mean±SE of five replicates.

In the earlier studies of *Agrobacterium*-mediated transformation of *Jatropha* using cotyledonary leaf explants reported integration of one to two copy (Kumar et al. 2008), single to multiple copies (Pan et al. 2010), and multiple copies of transgene (Li et al. 2007). The Southern data presented by Khemkladngoen et al (2011) was of very poor quality reflecting no evidence of transgene integration.

The three stable transgenic T_0 lines of *Jatropha* subjected to Cry1Ac protein expression analysis by rapid qualitative immunodiagnostic assay and ELISA. The clear appearance of test line in the DesiGen XpresstripsTM confirmed the presence of Cry1Ac

protein in T_0 transgenic lines (Fig. 7.5 a). The level of expression of Cry1Ac protein in transgenic *Jatropha* lines estimated through ELISA test showed the level of expression in the range of 0.001 to 0.089% of the total leaf soluble protein (Fig. 7.5 b). The results described above demonstrated that expression of the *cry1Ac* regulated by the double 35S-promoter led to the accumulation of Cry1Ac protein in transgenic plants.

7.3.4 Effect of Cry1Ac protein expression on agronomic traits of the plant

We monitored the effect of expression of Btcry1Ac on phenotype of Jatropha transgenic lines (T0). Three transgenic *Jatropha* lines and three control wild-type were studied for the plant height, number of primary branches, diameter of main stem, fruit per tree and seed per fruit after transferring the plant to soil (December 2009 to December 2012).

Table 7.1 Comparison of agronomic traits between wild-type and Jatropha transgenic lines

Plant	Height (cm)	Diameter of main stem (mm)	Number of primary branches	Fruit per tree	Seed per fruit
WT	325±9.2	29±3.05	3+0.5	40±0.5	3±0.1
Jat T_0	250±7.2	23±1	4+0.5	35±1	3±0.16

Recorded in Dec 2012. The data are presented as mean±SE, where WT: Wild-type Jatropha; Jat T_0 is transgenic Jatropha line expressing Bt*cry1Ac*.

The plants took around two years for flowering and fruiting. The preliminary data collection in first year of fruiting, *Jatropha* transgenic lines with average plant height of 250 cm and 35 fruits per tree did not show any negative effects on the above mentioned agronomic traits (Table 1).

7.4 Conclusion

Advancement of molecular genetics in biofuel crops for crop improvement requires efficient and reliable transformation systems that produce stable transgenic plants preferably with single copy of transgene integration and low frequencies of tissue

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culture-induced phenotypic abnormalities in the transgenic plants. In our study, we demonstrated the reproducibility of the improved *Agrobacterium* mediated transformation protocol in *Jatropha* through the effective integration of *cry1Ac* as proof of concept. The protocol was effective to the extent of recovery of transgenic lines with single copy transgene insertion through a stringent kanamycin selection regime. This is the first report on development of transgenic *J. curcas* expressing an insecticidal gene, *cry1Ac*. Furthermore, we demonstrate for the first time in *Jatropha* the evidence of maturity of transgenic plants and expression of transgenes in reproductive tissues and fruits. Although the expression levels by ELISA test showed high accumulation of Cry1Ac protein, the ongoing experiments on insect bioassay at green house level would provide a good opportunity to analyze the impact of *cry1Ac* for resistance to target insects in transgenic *Jatropha* lines with single copy of *cry1Ac* integration. In the present study, the T_0 plants developed following the improved protocol showed good recovery and normal growth under greenhouse conditions. Ongoing experiments on molecular analysis in progenies would determine the inheritance pattern and stability of the transgene in parental transgenic lines. The indicative experiments in the development of transgenic *Jatropha* formed the basis for our hypothesis of examining the stability of the transgenes in *Jatropha* using a validated gene controlling an agronomic trait. In this direction, *Jatropha* transgenic lines with single copy insertion events of *cry1Ac* would provide useful information about the utility of the *cry1Ac* in conferring resistance against target insects including *S. litura.* The use of insecticidal *cry* genes has been successfully demonstrated for insect pest management in the majority of the crops including oil seeds. Our improved transformation protocol will also facilitate the genetic improvement of this biofuel crop through manipulation of oil biosynthesis genes which could have positive implications on reducing the dependence on fossil fuel.

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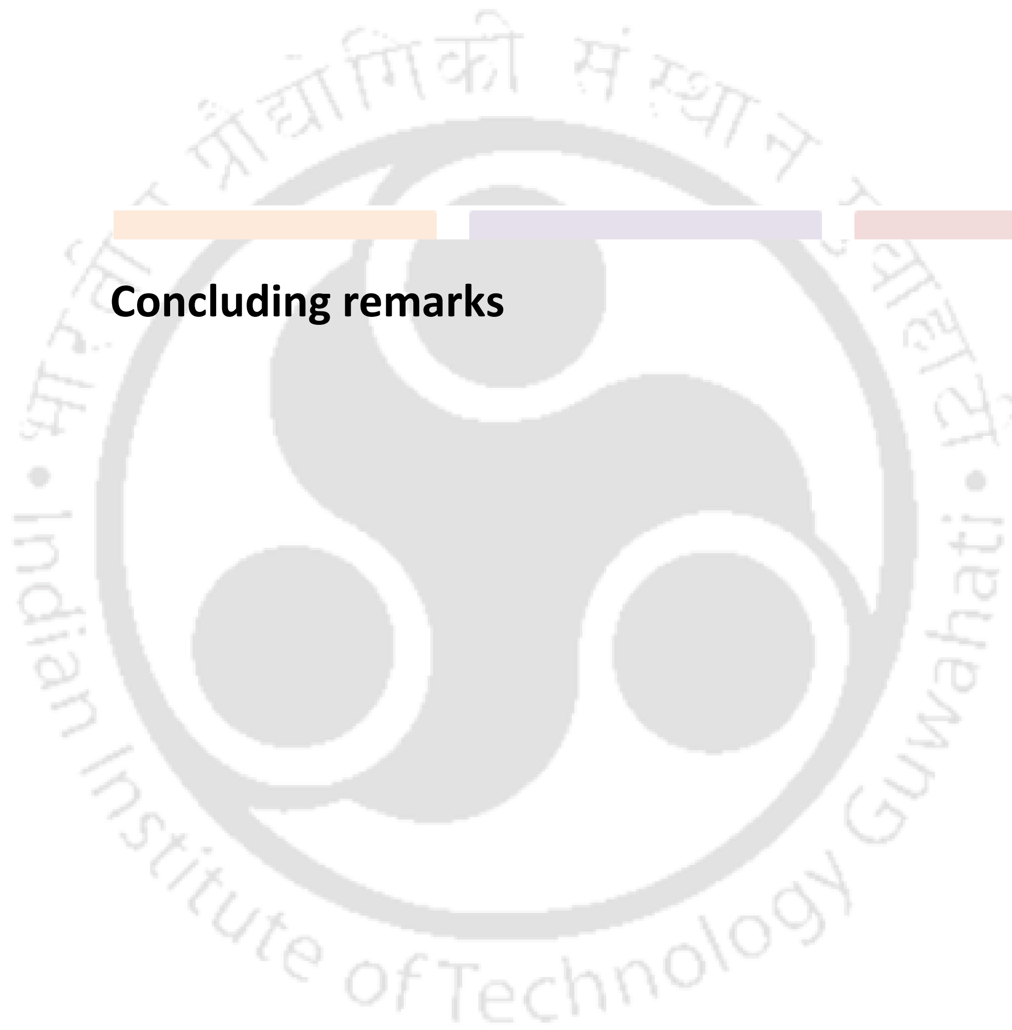

Concluding remarks

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8.1 Significance and salient features of the study

Being a non-edible high oil yielding plant, *Jatropha* has emerged as a promising biodiesel feedstock. It is believed that *Jatropha* can be used to alleviate the energy crisis, and is becoming one of the world's key crops for biodiesel production. However, *Jatropha* must not directly compete with food crops due to possible food crisis and limited farmlands in the world, so it must be resistant to a diseases and high degree of aridity. *Jatropha* can therefore be used to rehabilitate wastelands and improve the environment. It can also enhance the quality of rural life by providing new economic resources for marginal farmlands. However, the large scale profitable cultivation of *Jatropha* has not gained momentum due to low and inconsistent yield and lack of identified elite germplasm. There is an immediate need to identify elite germplasm *vis-a-vis* to develop superior genotype for higher seed yield and oil content, earlier maturity, reduced plant height, resistance to pests and diseases, drought resistance/tolerance, higher ratio of female to male flowers and improved fuel properties.

In the present study, elite accessions of *J. curcas* collected from Assam, North East India were screened on the basis of their seed trait and oil content. Four high oil yielding varieties were identified on the basis of high oil content (37.6-46.6%). Analysis of physico-chemical properties of the oil and biodiesel obtained from seeds of these elite germplasm demonstrated that they were within acceptable range of standards specifications of ASTM D6751, indicating a promising source for biodiesel feedstock from North East India. Furthermore, Life cycle assessment of *Jatropha* based biodiesel production showed cultivation process of *Jatropha* contributing to the highest environmental impact as compared with other stages of its life cycle due to the ineffective management of its cultivation. However, a comparison of *Jatropha* and *Pongamia* based biodiesel showed the superiority of *Jatropha* as a potential feedstock than *Pongamia*. In order to develop an insect pest resistant variety of *Jatropha* through genetic engineering, an efficient *de novo* plant regeneration system from cotyledonary

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leaf segment explants amenable to genetic manipulation through *Agrobacteium*-mediated transformation was established. The factors influencing the *Agrobacterium*-mediated *Jatropha* transformation such as, age of the explants, pH of the cocultivation media, acetosyringone concentration, cocultivation time and temperature were optimized. An efficient *Agrobacterium*-mediated transformation system in *Jatropha* was developed and transgenic *Jatropha* expressing insecticidal gene, *cry1Ac* were generated for the first time employing the developed transformation system. The transgenic *Jatropha* lines with single copy of *cry1Ac* integration generated in this study may provide a good opportunity to analyze the impact of *cry1Ac* for resistance to target insects. The improved transformation system established in this study would enable transfer of a wide range of candidate genes in *Jatropha* which in turn speed up the process of *Jatropha* varietal improvement program.

The salient features of the present study are summarized below:

- Elite accessions of *Jatropha* were identified by systematic collection, screening and selection of germplasm of Assam on the basis of their seed trait and oil content.
- Physicochemical properties of the oil and biodiesel obtained from the elite accessions showed the suitability of these accessions as potential feedstock for biodiesel production.
- Life cycle assessment of *Jatropha* biodiesel production indicated its cultivation process generating highest environmental impact as compared to other stages of its life cycle, and showed higher sustainability of biodiesel from *Jatropha* as compared to *Pongamia*.
- An efficient *de novo* plant regeneration system from cotyledonary leaf segment explants amenable to genetic manipulation through *Agrobacteium*-mediated transformation was established.

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- The critical factors affecting efficient gene transfer to cotyledonary leaf segment explants through *Agrobacterium*-mediated transformation were optimized.
- An insecticidal gene, *cry1Ac* was introduced to *Jatropha* and transgenic lines expressing *cry1Ac* were generated.

8.2 Future prospects

J. curcas is an important non-edible oilseed crop receiving worldwide attention as a potential biodiesel feedstock. The four elite *Jatropha* accessions identified in the present investigation formed the basis of varietal improvement program of *Jatropha* germplasm of Northeast India. Further, extensive survey of *Jatropha* germplasm in Northeast India through multi-location evaluation would hasten the process of identification of candidate plus tress of *Jatropha.*

Single step alkali catalyzed transesterification although sufficient for biodiesel production, more extensive and experimental study need to be carried out to investigate combustion, emission characteristics and performance of *Jatropha* based biodiesel on DI-CI Engine. The process design and kinetics of *Jatropha* oil transesterification in a batch reactor and analysis in biodiesel-fuelled engine are required to establish *Jatropha* biodiesel as successful alternative fuel.

Life cycle assessment quantifies and verifies the energy efficiency and the environmental impacts of bio-diesel production from the life cycle point of view. A transparent and accurate LCA of biofuel crops considering all the ecological and economical output from cradle to grave will provide foundation for intensive evaluation of their ecological and economic sustainability.

Genetically engineering for inherent crop resistance to insect pests offers the potential for environment-friendly method of crop protection for sustainable agriculture. The transgenic *Jatropha* lines with single copy of *cry1Ac* integration generated in our study would provide a good opportunity to analyze the impact of *cry1Ac* for resistance to target insects. The improved transformation system established

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in this study would enable incorporation of a wide range of candidate genes in *Jatropha* which in turn speed up the process of *Jatropha* varietal improvement program.

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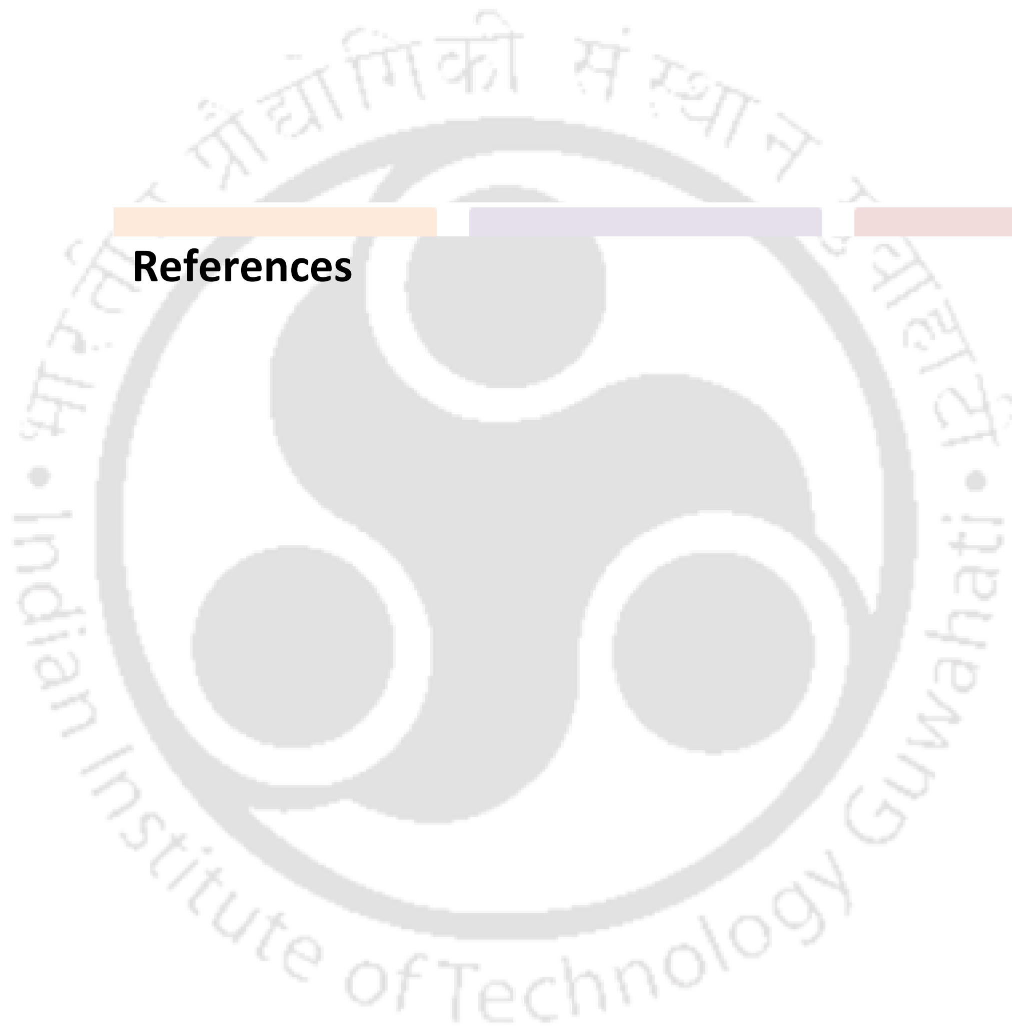

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List of Publications

Journals

1. **Mazumdar P,** Basu A, Paul A, Mahanta C, Sahoo L (2010) Age and orientation of the cotyledonary leaf explants determine the efficiency of *de novo* plant regeneration & *Agrobacterium tumefaciens*-mediated transformation in *Jatropha curcas* L. South Afri J Bot 76:337-344
2. **Mazumdar P,** Borugadda VB, Goud VV, Sahoo L (2012) Physico-chemical characteristics of *Jatropha curcas* L. of North East India for exploration of biodiesel. Biomass bioenergy 1-9
3. Purkayastha J, Sugla T, Paul A, Solleti SK, **Mazumdar P**, Basu A, Mohommad A, Ahme Z, Sahoo L (2010) Efficient *in vitro* plant regeneration from shoot apices and gene transfer by particle bombardment in *Jatropha curcas*. Biol Plantarum 54:13-20
4. Paul A, Thapa G, Basu A, **Mazumdar P**, Kalita MC, Sahoo L (2010) Rapid plant regeneration, analysis of genetic fidelity and essential aromatic oil content of micropropagated plants of Patchouli, *Pogostemon cablin* (Blanco) Benth.- An industrially important aromatic plant. Indus crop prod 32(3):366-374
5. Thounaojam TC, Panda, P, **Mazumdar P**, Kumar, D, Sharma GD, Sahoo, L (2012) Excess copper induced oxidative stress and response of antioxidants in rice. Plant Physiol Bioch 53:33-39

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1. **Mazumdar P**, Basu A, Purkayastha J, Mahanta C, Sahoo L (2008) Efficient regeneration from nodal explants of medicinal plant *Phyllanthus niruri*. International conference on Plant Biotechnology and Molecular Biology. 15th-17th August
2. **Mazumdar P**, Bas, A, Paul, A, Mahanta C, Sahoo L (2010) Age of the cotyledonary leaf explants of *Jatropha curcas* L. determines the efficiency of de novo plant regeneration and Agrobacterium tumefaciens-mediated transformation. National Symposium on Plant Cell, Tissue and Organ Culture: The Present Scenario, 3-5th March
3. **Mazumdar P**, Thapa G, Basu A, Sarma RN, Sahoo L (2011) Genetic variability and divergence studies in seed trait and oil content of *Jatropha curcas* in Assam, North East India. National Symposium on "Recent advances in plant tissue culture and

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Age and orientation of the cotyledonary leaf explants determine the efficiency of *de novo* plant regeneration and *Agrobacterium tumefaciens*-mediated transformation in *Jatropha curcas* L.

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Abstract

Effects of age and orientation of the explant on callus induction and *de novo* shoot regeneration from cotyledonary leaf segments of *Jatropha curcas* were studied. The callus induction and shoot regeneration capacity of cotyledonary leaf segments were found significantly related to the age of the explants and their orientation in culture medium. The youngest explant, derived from the cotyledonary leaf of germinated seed induced the highest regeneration response as compared to one- and two-week-old explants. A gradient response with age of the explant was observed in percentage of callus induction, shoot regeneration from callus and the number of shoots per regenerating callus. The explants cultured with their abaxial side in medium showed significantly higher regeneration response. The youngest explant was found to be most amenable to *Agrobacterium*-mediated transformation as compared to older explants. The fact that callus induced from the edges of the explant followed by *de novo* shoot induction, and strong transient *gus* expression observed in the edges of the explant are significant for routine *Agrobacterium*-mediated transformation and generation of stable transgenic plants in *J. curcas*.
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Keywords: Abaxial; Age; Cotyledonary leaf; *Jatropha curcas*; Multiple shoots; Transformation

1. Introduction

Jatropha (*Jatropha curcas* L.), a hardy perennial shrub member of the Euphorbiaceae has attracted global attention in recent past as an important source of biodiesel (Annarao et al., 2008). The seeds of Jatropha contain 30–40% oil with a fatty acid pattern similar to that of edible oils (Gubitz et al., 1999). The seed oil can be used as a diesel engine fuel as it has characteristics close to those of fossil fuel, diesel. The non-edible oil of Jatropha seeds and its derivatives are also used for manufacturing a number of useful products, including candles, high quality soaps, cosmetics, biopesticide and fertilizer as well as for healing several skin disorders (Kochhar and Kochhar, 2005). Jatropha can be grown in marginal wastelands due to its ability to adapt to adverse agroclimatic conditions (Kaushik et al., 2007). It has an estimated annual production potential of 200,000 metric tonnes in India (Tiwari et al., 2007). However, conventional propagation of Jatropha is limited by poor seed germination, scanty and delayed rooting of seedlings and vegetative cuttings (Heller, 1996; Openshaw, 2000; Purkayastha et al., 2010). The low germination of whole seeds indicates that whole seed germination under controlled conditions considerably understates the potential germinability of Jatropha. This has significant implications on seed bank determinations and predictive seedling recruitment estimates, which rely on whole seed germination methods to assess seed viability. In this context, embryo or embryo derived explants culture offers a useful solution as well as being a promising method for initiating stock cultures for micropropagation of Jatropha as the explants derived from field grown plants are

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associated with high degree of microbial contaminants, and less responsive to *in vitro* culture manipulation (Purkayastha et al., 2010). Furthermore, it could offer a rapid means for conservation of elite germplasm. Although, the use of this process for clonal propagation, demanding seed tissues as starting material, requires evaluation of the heterogeneity in clones use of seeds from inbred elite Jatropha lines could circumvent the screening requirement.

Despite the best protein composition of Jatropha seed press cake, its use as feed for livestock carries potential concerns due to the presence of toxic substances, including a lectin (curcin) and phorbol esters (Makkar et al., 1998). Increased use of Jatropha seeds as a fuel source would risk increase in the production of the toxic by-product (meal), which could pile up as a potential waste (Becker and Makkar, 1998). Genetic engineering appears to be an effective approach to reduce the levels of these toxic substances in seeds, and increase resistance to biotic stresses, and furthermore, offers opportunities to modify seed oil for higher engine efficiency (Hossain and Davies, 2010). However, the development of an efficient regeneration system amenable to transformation is a prerequisite for genetic manipulation of this important biofuel plant. Plant regeneration in *J. curcas* has been accomplished through organogenesis from various explants, including mature leaf (Sujatha and Mukta, 1996; Sujatha et al., 2005; Soomro and Memon, 2007; Deore and Johnson, 2008), petiole (Sujatha and Mukta, 1996), hypocotyls (Sujatha and Mukta, 1996; Soomro and Memon, 2007), axillary node (Sujatha et al., 2005; Shrivastava and Banerjee, 2008) and via somatic embryogenesis from mature leaf explants (Jha et al., 2007). However, amenability of these regeneration systems to genetic transformation methods has not been evaluated. Seed-derived explants are, in general, known to be more responsive to rapid regeneration (Pradhan et al., 1998; Tiwari and Tuli, 2009) and *Agrobacterium*-mediated transformation (Patnaik et al., 2006; Paz, 2009). Our laboratory has recently developed a method for rapid and efficient plant regeneration from shoot apices, and generation of transgenic plants by direct DNA delivery to mature seed-derived shoot apices of Jatropha (Purkayastha et al., 2010). However, *Agrobacterium*-mediated transformation is preferred as it offers several advantages, such as the defined integration of transgenes, preferential integration into transcriptionally active regions of the chromosomes, and potentially single or low copy number with rearrangement being relatively rare (Birch, 1997; Hiei and Komari, 2006). Therefore, to obtain a large number of independent transgenic Jatropha plants for screening over expression or cosuppression of candidate genes, a high frequency regeneration protocol amenable to *Agrobacterium*-mediated transformation is required. Report on *Agrobacterium*-mediated transformation system in Jatropha using cotyledonary leaf explants (Li et al., 2007) has proven to be either inefficient or difficult to reproduce, a proposition attributed to the inappropriate age of the explant used. The age of the explant is a critical aspect in transformation experiment and therefore, appropriate biological condition of the explant is vital for optimal infection and T-DNA transfer by *Agrobacterium tumefaciens* (Purkayastha et al., 2010). Furthermore, the choice of explants of appropriate age, and the orientation of the explants in culture medium have been indicated to exert significant influence on the frequency of plant regeneration (Thomas, 2003; Bhatia et al., 2005). As a first step to achieving this goal, we have evaluated the effect of the age, and orientation of the cotyledonary leaf explants on plant regeneration, and *Agrobacterium*-mediated transformation.

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2. Materials and methods

2.1. Plant material and explant preparation

Seeds of a local elite clone of *J. curcas* L. were collected from Rangia, Kamrup, Assam, India. The seeds were decoated and soaked in distilled water overnight at room temperature. The soaked seeds were treated with a 0.1% sodium hypochlorite solution containing few drops of Tween-20, for 10 min followed by washing under tap water for 20 min. The seeds were then surface sterilized with 70% alcohol for 5 min, and with 0.2% mercuric chloride for 10 min followed by four rinses in sterile double distilled water. After blot-dried on sterile filter paper, the endosperm was carefully dissected out to expose embryos with papery cotyledonary leaves. The papery cotyledonary leaves were separated out and cut into four segments (10 mm^2) with the edges removed and used as explants for callus induction, shoot regeneration and *Agrobacterium*-mediated transformation.

2.2. Induction of callus and shoot multiplication

The explants were placed with their abaxial surface firmly in contact with MS (Murashige and Skoog, 1962) medium supplemented with 6.66 µM of 6-benzylaminopurine (BAP) and 0.24 µM of indolebutyric acid (IBA) for 3 weeks in dark conditions for callus induction. The calli were transferred to MS medium supplemented with 6.66 µM BAP, 0.24 µM IBA and 1.44 µM gibberellic acid (GA_3) for induction of multiple shoots.

2.3. Influence of age and orientation of explants on shoot multiplication

To study the effect of the age of the explants on callus induction and shoot proliferation, and *Agrobacterium*-mediated transformation, cotyledonary leaves were obtained from freshly germinated seeds, and seedling developed from embryos, cultured for 1–2 weeks on MS medium. The leaves were cut into four segments (10 mm^2) with their edges removed to obtain the explants.

To investigate the effect of orientation of explants on callus induction and shoot proliferation, the cotyledonary leaf explants, and leaf explants (1–2 weeks old) were cultured with their abaxial or adaxial surface in firm contact with the medium.

2.4. Culture media and conditions

All culture media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar (Hi-media, Mumbai, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 15 psi and 121 °C for 20 min. All the cultures were maintained at 25 ± 2 °C under a 16 h photoperiod with a photosynthetic photon flux

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density of 35 μmol m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India).

After 3 weeks of incubation in the dark, the efficacy of each variant on callus induction, and after 4 weeks of incubation of calli in 16 h photoperiod, the shoot multiplication rate were determined by recording (1) the percentage of explants forming callus, (2) the frequency of shoot regeneration and (3) the average number of shoots per callus.

2.5. Shoot elongation, rooting and acclimatization

The individual shoots were separated from shoot clusters and transferred to MS medium supplemented with varied concentrations of GA_3 (0.5, 1.0, 1.5, and 2.0 μM) to allow for the elongation of shoots. Elongated shoots were cultured for rooting on half strength MS medium supplemented with varied concentrations (1.0, 2.5, and 5.0 μM) of IBA and α-naphthaleneacetic acid (NAA). Plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water, and transferred to plastic pots containing soil, vermiculite, and vermicompost (1:1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (28 °C day, 20 °C night, 16 h day-length, and 70% relative humidity). After a week, the plastic covering was gradually removed and the plantlets were maintained in the greenhouse in plastic pots containing normal garden soil until they were transplanted to the nursery.

Experiments were set up in a completely randomized design and each treatment had three replicates of 20 explants each. All data are statistically analyzed by ANOVA followed by Newman–Keul's multiple range test for mean comparison.

2.6. Transformation procedure and histochemical GUS-assay

A. tumefaciens strain EHA105 harbouring a binary vector pCAMBIA2301 which contains *β*-glucuronidase (*gus*) with an intron in the coding region and neomycin phosphotransferase (*nptII*) genes, both driven by CaMV 35S promoter was used for transformation studies. Single colony of the bacterial strain was inoculated in 25 ml of liquid AB minimal medium (Chilton et al., 1974) containing 10 mg/l rifampicin and 50 mg/l kanamycin, and grown overnight at 28 °C until OD_{600} reached to 0.8. The cells were collected by centrifuging at 5000 rpm for 5 min and the pellet was resuspended in liquid cocultivation medium, LCM (MS medium containing 6.66 μM of BAP and 0.24 μM of IBA, pH adjusted to 5.7) supplemented with 100 μM acetosyringone. The cotyledonary leaf explants excised from seeds, and leaf explants (1–2 weeks old) were inoculated in bacterial suspension for 30 min with occasional shaking in dark. The explants were then blotted on sterile filter paper and cocultivated in petridishes lined with filter paper, moistened with LCM supplemented with 100 μM acetosyringone for 3 days at 25 °C. After 3 days of cocultivation, the explants were washed three to four times with sterile distilled water and blotted dry on sterile filter paper.

Histochemical GUS assays (Jefferson et al., 1987) were used to assess transient expression of the *gus* gene. Transient *gus* expression in leaf explants was scored after 3-day-cocultivation, and the number of explants showing transient *gus* expression at their edges were scored by immersing the tissue materials in GUS substrate solution for 24 h at 37 °C. Following incubation, tissues were bleached with 100% ethanol, and examined under microscope.

3. Results and discussions

3.1. Callus induction and shoot multiplication

Cotyledonary leaf explants (Fig. 1a) placed on the MS medium supplemented with 6.66 μM of BAP and 0.24 μM of IBA exhibited distinct morphological changes. The explants turned to green in color within a week of culture, enlarged, and swelled at their edges within the following two weeks of incubation showing the sign of dedifferentiation (Fig. 1b). The explants formed white friable callus after 3 weeks of culture. *De novo* shoot bud induction was observed on the calli (Fig. 1c and d) developed at the edges of explants, within two weeks of transfer of calli to MS medium containing 6.66 μM BAP, 0.24 μM IBA and 1.44 μM GA_3 at 16 h photoperiod regime. The cut edges of leaves provided a way for nutrients and growth regulators to be absorbed efficiently from the medium (Sarwar and Skirvin, 1997), and the synergistic effect of BAP and IBA was shown to trigger response for callus induction in Jatropha (Sujatha and Mukta, 1996; Weida et al., 2003; Li et al., 2007). Prior incubation in the dark was a very important process for dedifferentiation and redifferentiation of Jatropha. Prior incubation in the dark has been reported to increase shoot regeneration in various plant species, such as Zhanhua winter jujube (Gu and Zhang, 2005), campanula (Sriskandarajah and Serek, 2004), watermelon (Compton, 1999), quince (Baker and Bhatia, 1993), pear (Chevreau et al., 1989), apple (Fasolo et al., 1989), and blueberry (Billings et al., 1988). Incubation in the dark may delay degradation of endogenous and/or exogenous plant growth regulators (Rusli and Pierre, 2001). In addition, dark treatment may reduce the levels of cell wall thickness and cell wall deposits (cellulose and hemicellulose), facilitating translocation of plant growth regulators in plant cells (Herman and Hess, 1963). The calli developed cluster of multiple shoots (Fig. 1e) with an average of 12.56 shoots in 94% of the responded calli (Table 1) within 4 weeks of transfer of callus to shoot induction medium. Cotyledonary leaves have been most widely studied for *de novo* shoot formation in diverse plant species (Nikam and Shitole, 1997; Amutha et al., 2003; Vengadesan et al., 2003).

3.2. Influence of age and orientation of explants on callus induction and shoot multiplication

The factors that significantly influenced the efficiency of callus induction and prolific *de novo* plant regeneration in cotyledonary leaf segment explants of Jatropha were the age of the explant, and its orientation in culture. Although the explants of different age formed callus and subsequently induced clusters of multiple shoots, however, the percentage of explants that formed callus, the percentage of callus that induced multiple shoots and the average number of shoots per callus significantly

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Fig. 1. (a–l). *De novo* shoot bud induction and plant regeneration from cotyledonary leaf explant of *Jatropha curcas* L. (a) Cotyledonary leaf segment explant of freshly germinated seed (bar: 0.7 cm). (b) White, friable callus induced from cotyledonary leaf segment on MS medium with BAP (6.66 μM) and 0.24 μM indole-3-butyric acid (IBA) after 3 weeks of dark incubation (bar: 1.4 cm). (c) *De novo* shoot bud induction from cotyledonary leaf segment derived callus on MS medium supplemented with BAP (6.6 μM), IBA (0.24 μM) and GA_3 (1.44 μM) after 2 weeks of culture at 16 h photoperiod regime (bar: 1.8 cm). (d) Microscopic view of leaf edge showing shoot bud induction (bar: 0.15 mm). (e, f) Profuse shoot bud clump formation from cotyledonary leaf segment derived callus. (g) Shoot elongation on MS medium supplemented with GA_3 (1.0 μM) after 2 weeks of culture. (h) Rooting of *in vitro* regenerated shoot on half strength MS medium supplemented with NAA (5.00 μM) within 17 days of culture. (i) An acclimatized plant. (j) Plants established in nursery. (k, l) GUS expression at the cut edges of cotyledonary leaf segment immediately after cocultivation with *Agrobacterium tumefaciens* EHA105pCAMBIA2301 (k) and untransformed control leaf explant (l).

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Table 1
Effect of age of explant on callus formation and multiple shoot induction from cotyledonary leaf explants of *J. curcas* L.

Age of the cotyledonary leaf explant (days)	Response of the cotyledonary leaf explant		
	Callus formation (%)	Average no of shoots	Regeneration frequency (%)
0	87.50^{a}±3.46	1256^{a}±0.38	87.50^{a}±3.11
7	75.83^{ab}±3.46	4.35^{b}±0.32	65.83^{b}±3.27
14	70.83^{bc}±3.46	2.66^{c}±0.35	57.50^{bc}±3.24

The values are the means (±SE) of six replicates with 20 explants each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman–Keul's multiple range test.

Table 2
Effect of orientation of explant in the culture medium on callus formation and multiple shoot induction from cotyledonary leaf explants of *J. curcas* L.

Orientation of the cotyledonary leaf disc attached to the medium	Response of the leaf disc		
	Percentage of callus formation	Regeneration frequency (%)	Average no of multiple shoots
Abaxial	87.84^{a}±4.32	87.62^{a}±4.06	12.54^{a}±0.38
Adaxial	54.23^{b}±3.63	56.34^{b}±4.45	07.35^{b}±0.42

The values are the means (±SE) of four replicates with 20 explants each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman–Keul's multiple range test.

declined with the increase in age of the explants (Table 1). The best regeneration response was observed in explants, prepared from cotyledonary leafs of freshly germinated seeds with 87.5% of the explants forming callus, out of which 94% of the callus regenerated forming an average of 12.56 shoots, a response which was significantly higher than one- and two-week-old explants (Table 1). Famiani et al. (1994) proposed that explants from young leaves show more regeneration potential than older leaves as the younger leaves, still developing, have less differentiated and more metabolically active cells, and therefore, under suitable hormonal and nutritional conditions show improved plant regeneration. Furthermore, explants of different age may have different levels of endogenous hormones and, therefore, the age of explants would have a critical impact on the regeneration efficiency, similar results have been reported in other plants, including *Platanus occidentalis* (Sun et al., 2009), *Morus alba* (Thomas, 2003), *Cajanus cajan* (Dayal et al., 2003), *Rosa hybrida* (Ibrahim and Debergh, 2001), *Aerides maculosum* (Murthy and Pyati, 2001), *Cercis canadensis* (Distabanjong and Geneve, 1997), *Malus* (Famiani et al., 1994), *Cydonia oblonga* (Baker and Bhatia, 1993), *Aegle marmelos* (Islam et al., 1993), *Lachenalia* (Niederwieser and Van Staden, 1990) and *Prunus* (Mante et al., 1989). In contrast with the above results, a higher regeneration response was observed from explants of older leaves as compared to the younger explants in apple (Antonelli and Druart, 1990).

Success of regeneration from leaf explants depends not only on the age of the explant chosen, but also the way explants are placed on the culture media (Duzyaman et al., 1994; Bhatia et al., 2005). Explants can be inoculated on the culture media in abaxial (lower surface facing down) or adaxial (upper surface facing down) orientation for the cotyledons and leaves (George, 1993). The best results were obtained when the leaf abaxial side was touching the culture medium as in this position the regeneration obtained was nearly two-fold higher than the leaf explants cultured with their adaxial side in contact with the medium (Table 2). Similar observations have been reported with the leaf abaxial side touching the culture medium (Dolcet-Sanjuan et al., 1990; Fiola et al., 1990; Leblay et al., 1990; Stamp et al., 1990; Welander and Maheswaran, 1992; Duzyaman et al., 1994; Bartish and Korkhovoi, 1997). Shoots produced from the adaxial orientation protruded into the culture medium and then turned upwards, trying to emerge from the medium. This resulted in a reduced number of shoots being produced per explant. The contrasting response of the explants in its two different orientations is attributed to the total surface area of the explants that come in contact with the medium.

3.3. Elongation of shoots

In the present study, shoot clumps produced on caulogenesis medium, either took prolonged duration for elongation of a few shoot buds or majority of the shoots buds turned necrotic and later died on transferring to phytohormone-free basal medium, a response which is quite prevalent in plant regeneration from callus (Ibrahim and Debergh, 2001). It appears that the hormone levels present in the medium used for shoot induction have a carry-over effect in the shoots. This situation caused prolific multiplication of shoot initials from the calli, and this process prevented individual shoot elongation. It was necessary to develop a suitable medium for faster elongation of shoot buds. Incorporation of 1.0 µM GA_3 to MS media significantly enhanced the shoot elongation within one to two weeks of culture (Table 3; Fig. 1g), as compared to medium devoid of GA_3. The promotive effect of GA_3 on elongation of stunted shoots has been reported in several other plant species (Demeke and Hughes, 1990; Jordan and Oyanedel, 1992; Purohit and Singhvi, 1998; Sugla et al., 2007; Purkayastha et al., 2008). GA_3 is considered to stimulate shoot elongation by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger, 1998).

3.4. Rooting and transplantation

Rhizogenesis is the final step in the formation of complete plantlet in *in vitro* culture. Root formation occurred in 40% of the shoots within 20–22 days of culture on half strength MS medium.

Table 3
Effect of gibberellic acid (GA3) on elongation of shoots from cotyledonary leaf derived callus of *J. curcas* L. on MS medium after two weeks of culture.

GA3 (µM)	Mean shoot length (cm)	Fold increase in shoot length
0	1.37^{a}±0.07	0
0.5	2.90^{b}±0.03	1.11^{b}±0.03
1.0	5.12^{c}±0.03	2.73^{a}±0.03
1.5	3.22^{d}±0.03	1.35^{b}±0.03
2.0	3.35^{e}±0.03	1.44^{b}±0.03

The values are the means (±SE) of three replicates with 20 cultures each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman–Keul's multiple range test.

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No roots could be induced on full strength MS basal medium, wherein the shoot bases turned brown, indicating necrosis effect. Roots were not induced on medium containing IBA, and the shoots formed varying degree of callus at shoot base (Table 4). However, NAA containing medium induced roots without any callus induction at the shoot base (Table 4). Highest percentage of root induction occurred in 75% of the shoots in the medium containing 5.0 μM NAA within 17–20 days of culture (Fig. 1h). The contrasting difference between the present observation and our previous report (Purkayastha et al., 2010), wherein all the shoot apex-derived shoots induced roots in half strength medium could be attributed to the origin of shoots. Plantlets with well-established roots were successfully acclimatized (Fig. 1i) in pots containing soil, vermiculite and vermicompost (1:1:1) and eventually established in a nursery (Fig. 1j) with survival frequency of 97%. The established plants were apparently uniform and did not show any observable variation.

3.5. Influence of age of explants on Agrobacterium-mediated transformation

Agrobacterium-mediated transformation is the preferred method for its simplicity, cost effectiveness, little rearrangement of transgenes, ability to transfer relatively longer DNA segments (Hamilton et al., 1996) and preferential integration of foreign genes into transcriptionally active regions (Ingelbrecht et al., 1991) ensuring thereby proper expression of transgenes in transgenic plants (Hernandez et al., 1999). However, *Agrobacterium*-mediated transformation has often proved unsuccessful due to the accumulation of secondary metabolites in explants used, and particularly complex mixtures such as the oils (Sugimura et al., 2005) or bacteriostatic polyphenols (Kumar et al., 2004). Therefore, selecting leaves at a developmental stage that possesses low amounts of oil, without compromising their prolific regeneration ability of the explants is crucial for establishing successful *Agrobacterium* infection and transgenic plant generation. In order to select leaf materials for their greater amenability to *Agrobacterium*-mediated genetic transformation, the transient *gus* expression analysis was performed on explants from cotyledonary leaves of different age, following three days of cocultivation with *A. tumefaciens*. The cotyledonary leaf disc explants excised from one- and two-week-old *in vitro* raised seedlings were compared with cotyledonary leaf disc explants excised from freshly germinated seeds for their transformation efficiency. Strong *gus* expression was observed at their edges (Fig. 1k), and the explants demonstrated ability for *de novo* shoot formation. The endogenous *gus* activity (color) was not detected in non-transformed (control) explants (Fig. 1l). The *gus* activity at the cut ends indicated the susceptibility of explants to *Agrobacterium*-mediated transformation. However, a significant difference in percentage of explants showing *gus* expression was observed among the explants of different ages (Fig. 2). The number of *gus* expressing explants was significantly higher in the case of cotyledonary leaf explants, excised from the freshly germinated seeds, as compared to the explants excised from one- and two-week-old leaves (Fig. 2).

In conclusion, the age of explants, and its orientation in culture medium were found as the most critical factors influencing induction of callus and *de novo* shoot regeneration from cotyledonary leaf explants in *J. curcas*. The data presented in this report clearly suggested that the cotyledonary leaf segment explants prepared from seeds, cultured with their abaxial surface placed in the medium showed significantly better regeneration response as compared to older explants, and the explants cultured with their adaxial surface in culture medium. Furthermore, cotyledonary leaf segment explants, prepared from freshly germinated seeds were found to be most amenable to *Agrobacterium*-mediated transformation as compared to older explants. The method established here could be employed for routine transformation by *Agrobacterium*-mediated method for transformation of cotyledonary leaf explants and *de novo* generation of transgenic plants in *J. curcas*.

Table 4
Effect of different concentrations of auxins, IBA and NAA added to half strength MS medium on rooting of shoots raised from cotyledonary leaf derived callus of *Jatropha curcas* L.

Auxin (μM)	Percentage of rooting	Degree of callus formation at shoot base	No of days required for rooting
–	40^{a}±3.71	–	20–22
IBA			
1.0	–	+	–
2.5	–	++	–
5.0	–	++++	–
NAA			
1.0	–	–	–
2.5	55^{b}±4.26	–	19–22
5.0	75^{c}±4.39	–	17–20

The values are the means (±SE) of three replicates with 20 cultures each. Means within a column followed by different letters are statistically significant at $P<0.05$ by Newman-Keul's multiple range test.

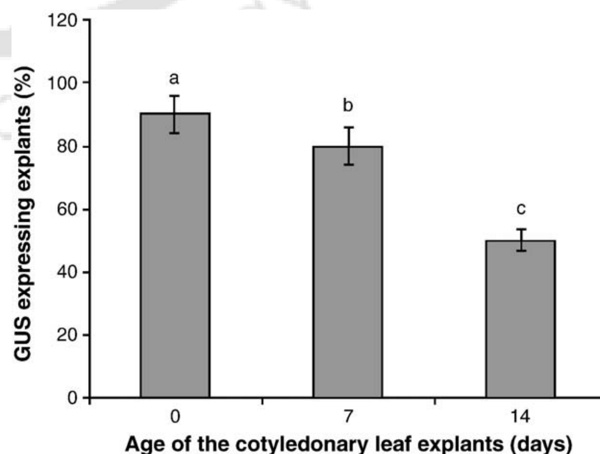

Fig. 2. Effect of age of the explant on efficiency of T-DNA transfer in *Agrobacterium*-mediated transformation of cotyledonary leaf explants of *J. curcas* L. Different letters denote significant differences at $P<0.05$ between treatments. Bars represent standard errors.

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Physico-chemical characteristics of *Jatropha curcas* L. of North East India for exploration of biodiesel

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ABSTRACT

Oil content of wild genotypes of *Jatropha curcas* L. collected from Assam, North East India, was studied. A total of 20 genotypes were used to investigate the oil content and variation in the oil content was found to be between 23.70 and 46.60%. Among them four high oil yielding genotypes was selected. Physicochemical properties of the oil and biodiesel obtained from those four genotypes were analyzed to check the suitability of these genotypes as potential candidate for biodiesel production. The gas chromatographic (GC) analysis showed amount of total monounsaturated fatty acid (oleic acid) is more in comparison to polyunsaturated fatty acid. ^{1}H NMR profile of the oil and biodiesel demonstrated the successful conversion to methyl ester. The thermogravimetric analysis (TGA) under nitrogen and oxygen environment revealed the thermal and oxidative stability of oil and methyl esters. The obtained results were found to be within acceptable range of standards specifications of ASTM D6751, showing a promising source for biodiesel production in North East India.

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1. Introduction

Gradual dwindling of world fossil reserves and emissions of green house gasses are leading to energy insecurity and ecological imbalance in a foreseeable future. Biodiesel derived from vegetable oils seems to be a solution as it is renewable and ecofriendly in nature. Biodiesel is basically monoalkyl esters of fatty acids produced from vegetable oils or animal fats by transesterification with small chain alcohols [1]. Currently, more than 95% biodiesel are produced from edible oil feedstock [2]. This will make biodiesel economically unfeasible as compared to petroleum-derived fuels [3,4]. To avoid this situation, non-edible oil seeds need to be used for commercial production of biodiesel. Many researchers have initiated work on the use of low cost non-edible oils as alternative feedstock for biodiesel production [5–7]. Among non-edible oil feedstock, seeds of *Jatropha curcas* L. proved to be a highly promising reliable source having high seed oil content. Although the species is native to Central America is now widely distributed across the World [8]. *J. curcas* oil is non-edible due to presence of toxic phorbol esters and curcin [9].

Assam is a central state in North East India and it is one of the “biodiversity hotspot” of the world. The species *J. curcas* is now well naturalized and became common flora in this unique bio geographic region. Recently, government of Assam

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has initiated State-level Jatropha Mission for the plantation of this biofuel crops in forest and wasteland through Forest Development Agencies, self-help groups and other institutions. The main objective of this step is to provide employment opportunity to people and enhance the quality of rural life. Current estimates suggest that there are now 40 ha of *J. curcas* planted in Assam with the target of additional 2000 acres to be planted. However, this large scale Jatropha plantation might hold the risk of unsustainable practices and loss of resources. This risk is mainly due to the lack of proper scientific knowledge and technology about the oil content and feasibility of this oil for production of fuel.

So far, there is no report in the literature on screening and identification of *J. curcas* from North East India for biodiesel production. Hence, there is an urgent need to investigate the oil content and physico-chemical properties of *J. curcas* in Assam for judicious utilization of resources. Therefore, in this paper effort has been made to analyze oil content and fuel properties of Jatropha collected from different zones of Assam, for exploration of potential biodiesel sources.

2. Materials and methods

2.1. Materials and reagent

A through and extensive survey was conducted to collect seeds from different location of Assam (Fig. 1). The selection was based on extensive phenotypic assessment of qualitative characters. The seeds were separated from the fruit mechanically and cleaned manually to remove all foreign material. The cleaned seeds were dried under similar temperature (60 °C) and humidity conditions to reach constant weight. Pure standards of FAME and Wijs solution were purchased from M/s Sigma Aldrich Company (St. Louis, MO). All other chemicals and reagents (methanol, ethanol, n-hexane, potassium hydroxide, sodium thiosulphate, soluble starch, and phenolphthalein indicator were analytical reagent grade and purchased from M/s Merck.

2.2. Extraction procedure of J. curcas seed oil

One hundred grinded kernels were used to extract oil in three replicates. Oil was extracted in soxhlet apparatus using hexane as per the standard AOAC (American Oil Chemical Society) procedure for 8 h. The extract was concentrated in rota vapor, the residual oil was cooled and weighed. The physico-chemical properties of the oil were determined.

2.3. Transesterification of J. curcas seed oil

Single step direct transesterification procedure was followed due to low acid value of the native oil. Transesterification reaction was carried out in 250 ml special glass vessels (3 mm thick) sealed tightly and fitted with condenser at the top. The reaction glass vessel was placed on the hot plate magnetic stirrer. Methyl esters of Jatropha seed oil were prepared by refluxing the oil at 60 °C employing a 1:6 molar ratio of oil to methanol for one and half hour with 1 wt% KOH as catalyst and the mixture was stirred using a magnetic beads (400 rpm) [10]. After completion of the reaction, the mixture was cooled to room temperature and poured in a separation funnel, leading to separation of two phases. The bottom glycerol layer was discarded and the top ester layer was washed gently several times with warmed millipore water to remove the trace of catalyst, glycerol, and soap. A pH meter was used to check the complete removal of the catalyst. The washed

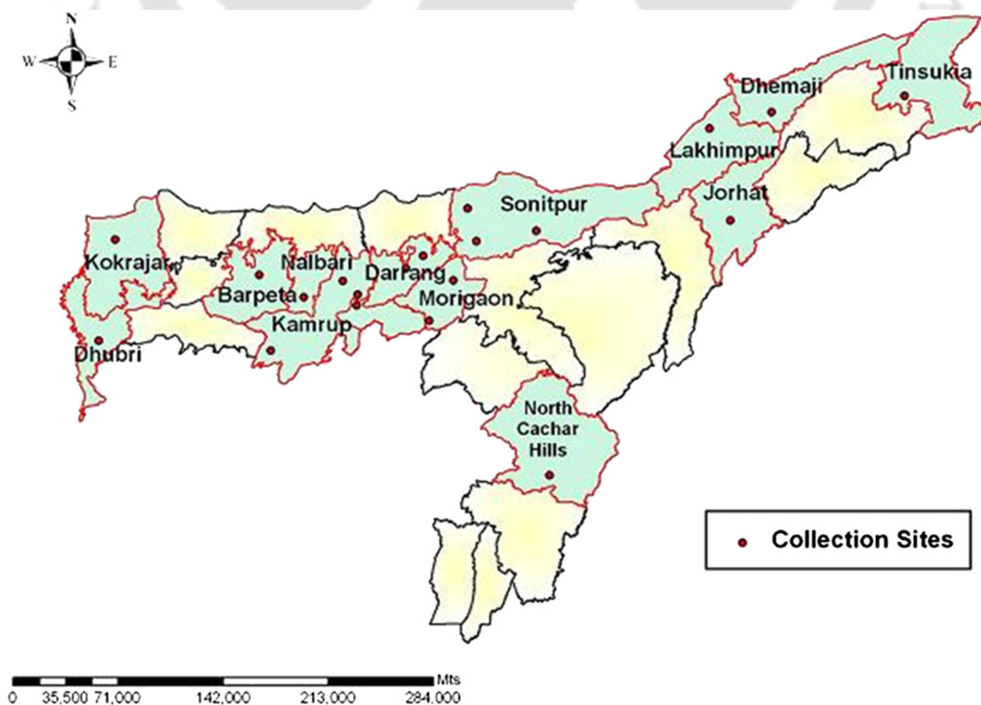

Fig. 1 – Map showing the collection sites of *Jatropha curcus* L. accessions represented in this study.

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methyl ester was further purified under vacuum on a rotary evaporator.

2.4. Analysis of *J. curcas* methyl esters (FAMEs)

The fatty acid composition of the methyl esters was determined using a Gas Chromatography (GC, CP-3800, Varian, Netherland) instrument, equipped with a capillary injection system operating at 200 °C, with a split ratio of 80:1 and a 1 μl sample size. A fused capillary column (EC Wax with 0.25 mm internal diameter, 30 m length and 0.25 μm film thicknesses) was used. The detection system was equipped with a flame ionization detector (FID). The detector temperature was programmed at 250 °C with flow rate of 2 ml/min. The carrier gas was high-purity nitrogen gas. The GC analysis was carried out at column temperature program consisted of initial temperature of 160 °C, 2 °C/min from 150 to 230 °C; increase at 4 °C–250 °C and hold for 5 min. The identification of the peaks was achieved by comparing its retention time to the retention time of standards analyzed under the same conditions The percentage of peak area of each fatty acid to the total peak area of all the fatty acids in the oil sample was used to calculate relative percentage of each fatty acid component in the methyl esters. The fuel properties of *J. curcas* oil and the fatty acid methyl esters (FAMEs) were determined as per the ASTM (D6751) standards.

2.5. ^{1}H NMR spectroscopy

^{1}H NMR spectrum of *J. curcas* oil and the fatty acid methyl esters was obtained on 500 MHz NMR spectrometer (Oxford, AS400, China) using a 5 mm broad band inverse probe head, equipped with shielded z-gradient accessories. Samples were dissolved in 400 μl deuterated chloroform ($CdCl_3$) and transferred to the 5-mm NMR tube. The deuterated chloroform chemical shift peak at 7.26 ppm was taken as internal reference. Typical parameters used were: spectral width: 4800 Hz; time domain data points: 32 K; flip angle: 90 °C; relaxation delay: 5 s; spectrum size: 32 K points; and line broadening for exponential window function: 0.3 Hz.

2.6. Thermogravimetric analysis (TGA)

The thermogravimetric profile of *J. curcas* oil and its methyl esters was obtained using Thermogravimetric Analyzer (TGA 851e/LF/1100, Mettler, Switzerland) at the heating rate of 10 °C/min in both nitrogen and oxygen atmosphere. The sample size was kept almost same 6 ± 0.5 mg throughout the study.

3. Results and discussion

3.1. Oil extraction

Geographical location (Fig. 1) and agro-climatic condition of 20 different location of Assam, from where seeds were collected represented in Table 1. For the study, from each wild genotype 100 seeds were drawn randomly in three replicates (total 300 seeds). Seed coats were removed and morphometric analysis was performed (Table 2). A comparison of mean performance revealed that maximum seed length was observed in IITJ13 (17.80 mm) and minimum in IITJ20 (14 mm). Maximum seed breadth was found in IITJ11 (11 mm) and minimum in IITJ20 (7.8 mm) respectively. Seed thickness varied from minimum 4.60 mm in IITJ20 to maximum 8.00 mm in IITJ14. Hundred

Table 1 – Geographical locations (latitude and longitude and altitude) and overall agro-climatic conditions (annual rainfall, annual temperature) of the different collection place of *Jatropha curcas* L. represented in this study.

Sl. no	Sample no	Collected from	Latitude	Longitude	Altitude (m)	Annual rainfall (mm)	Annual temperature °C (min–max)
1	IITJ1	Kamrup-1 (Amingaon)	26° 16′ 60N	91° 40′ E	31.00	1600	7–38
2	IITJ2	Kamrup-2 (Boko)	25° 58′ 60 N	91° 16′ 0 E	112.00	2234	7–38.5
3	IITJ3	Kamrup-3 (Changsari)	26° 16′ 60N	91° 42′ E	34.00	2230	7–38
4	IITJ4	Kamrup-4 (Rangia)	26.47′ N	91.63′E	38.00	1900	8–37
5	IITJ5	Barpeta	26° 19′N	91° E	34.00	2290	30–35.5
6	IITJ6	Dhemaji	27° 26′ 07″ N.	94° 39′ E.	104.00	4729	5–39.9
7	IITJ7	Darrang-1 (Kharupetia)	26° 31′ 11″ N	92° 7′ 47"E	36.90	1790	10–30
8	IITJ8	Darrang-2 (Mangaldoi)	26.43°N / 26.43; 92.03	92.03°E	36.80	1810	10–30
9	IITJ9	Dhubri (sadar)	26° 02′N	89° 58′E	35.00	2560	7–36
10	IITJ10	Jorhat	26° 45′N	94° 13′E	91.00	2270	7–28
11	IITJ11	Kokrajhar	26° 24′N	90° 16′E	37.00	3714	10–36
12	IITJ12	Morigaon-1 (kopahara)	26°50′N	92° E	60.60	2296	10–38
13	IITJ13	Morigaon-2 (Jagiroad)	26.4° N	92.5° E	60.60	2000	10–37
18	IITJ14	Nalbari (Chamota)	26°25′N	91° 26′E	41.00	2420	10–35
15	IITJ15	North Cachar Hills (Haflong)	25°11′N	93° 02′E	512.00	2270	10–30
14	IITJ16	Lakhimpur	27°14′N	94° 07′E	87.00	3350	8–32
16	IITJ17	Sonitpur-1 (Bihuguri)	26° 40′ N	92° 42′ E	60.00	2393	18.1–27
17	IITJ18	Sonitpur-2 (Dhekiajuli)	26° 42′N	92° 30′E	99.00	1200	16–29
19	IITJ19	Sonitpur-3 (Singri)	26° 36′ N	92° 28′ 60E	42.00	1210	16–28
20	IITJ20	Tinsukia (Makum)	27° 30′ 0N	95° 27′ 0E	121.00	2000	7–34

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Table 2 – Variability in seed morphology and oil content in *Jatropha curcas* L.

Sl. no.	Sample no	Seed length (mm)	Seed breadth (mm)	Seed thickness (mm)	100-seed weight	Oil content (%)
1	IITJ1	15.80	8.80	7.4	41.62	34.80
2	IITJ2	15.93	9.00	7.2	43.61	36.80
3	IITJ3	16.50	8.97	6.85	48.99	34.80
4	IITJ4	14.30	8.60	7.23	46.62	30.23
5	IITJ5	16.50	9.00	7.50	38.94	34.17
6	IITJ6	15.00	8.00	6.0	50.90	27.87
7	IITJ7	15.00	9.00	5.83	54.40	36.91
8	IITJ8	14.67	9.00	5.67	53.97	37.63
9	IITJ9	15.50	9.00	7.00	48.99	38.32
10	IITJ10	15.60	9.00	6.00	52.30	34.33
11	IITJ11	15.00	11.00	6.33	56.77	23.70
12	IITJ12	15.16	9.00	7.20	45.60	32.49
13	IITJ13	17.80	9.40	7.80	48.91	33.27
14	IITJ14	16.60	9.00	8.00	45.60	36.03
15	IITJ15	16.40	9.00	7.02	50.30	28.87
16	IITJ16	15.00	10.00	7.00	56.12	35.13
17	IITJ17	16.83	10.00	7.00	50.09	30.93
18	IITJ18	16.00	9.00	7.50	50.37	39.30
19	IITJ19	16.67	9.67	7.17	50.11	35.17
20	IITJ20	14.00	7.80	4.60	36.57	46.60
C.V.		0.06	0.07	0.12	0.11	0.13
SE (±)		0.21	0.15	0.19	1.20	1.07

seed weight varied from 36.57 to 56.77 g. Oil content varied from 23.70% (IITJ11) to 46.60% (IITJ20). During the study it was observed that among all the collected genotypes IITJ8, IITJ9, IITJ118 and IITJ20 found to have more oil content (Table 2).

3.2. *Fatty acid profile of J. curcas oil*

Fatty acid composition of a biodiesel demonstrates its potentiality as efficient fuels for future generation. The carbon chain length and the degree of saturation and unsaturation of the fatty acid molecules modulate the fuel properties of a biodiesel [11]. Generally, fatty acids present in a triglyceride are saturated (SFA, Cn:0), monounsaturated (MUFA, Cn:1) and or polyunsaturated with two or three double bonds (PUFA, Cn:2,3). The cetane number (CN), which is an indicator of the ignition quality of biodiesel, decreases as carbon chain length decreases and branching increases [12]. Long chain SFA increases the cloud point, cetane no, improves oxidative stability and reduces NOx emission but more PUFA reduce the cloud point, cetane no, stability and increases NOx emission [13,14]. As the requirements are antagonistic between cold flow properties in one hand and oxidative stability, NOx emission and the cetane number on the other, there is no fatty acid profile that will provide all optimal fuel parameters [15]. However, a good compromise can be reached by using vegetable oil having low saturated and polyunsaturated fatty acid. So, the vegetable oil with higher monounsaturated fatty acid was found to be a promising alternative to fossil fuels [16].

The fatty acid profile of the methyl esters of four high oil yielding genotypes of *J. curcas* are determined by GC and presented in Table 3. The main component are palmatic ($C_{16:0}$), stearic, oleic ($C_{18:0}$), linoleic ($C_{18:2}$) and linolenic acid ($C_{18:3}$). The fatty acid composition is dominated by the oleic acid (MUFA) followed by linoleic acid (PUFA) (Table 3). The oleic acid content was found higher in biodiesel obtained from IITJ20 (46.1%) compared to IITJ8, IITJ9 and IITJ18. Biodiesel obtained from IITJ8 seeds shows highest percentage of linoleic acid (35.2%) compared to other three genotypes. The presence of this polyunsaturated fatty acid in the Jatropha oil is expected to impart semi-drying property and can be used in surface coating industries [17]. Besides this, Table 3 shows that IITJ20 has higher amount of unsaturated fatty acid than other three genotypes. But the difference in fatty acid composition is negligible. So, the fatty acid profiles of all four accessions rich in oleic acid depicted a promising alternative to diesel fuel in future. Besides this, NOx emission because of high MUFA in Jatropha biodiesel can be reduced by using preheated biodiesel with penalty of little smoke emission [18].

3.3. *Physiochemical characterization of J. curcas oil*

The physico-chemical characteristics of oils were estimated as per the ASTM standard methods and presented in Table 4. Initially, the specific gravity of the oil samples was determined using the standard method mentioned above. Specific gravity of IITJ8, IITJ9, IITJ18 and IITJ20 was found to be 0.868, 0.867, 0.866 and 0.866 respectively, which is within acceptable range

Table 3 – Fatty acid profile *Jatropha curcas* L. methyl esters.

Fatty acids	Formula	Structure	Fraction (%)			
			IITJ8	IITJ9	IITJ18	IITJ20
Palmitic acid	$C_{16}H_{32}O_2$	16:0	12.7	11.5	11.9	11.4
Stearic acid	$C_{18}H_{36}O_2$	18:0	7.9	7.8	7.7	7.8
Oleic acid	$C_{18}H_{34}O_2$	18:1	41.48	44	43.3	46.1
Linoleic acid	$C_{18}H_{32}O_2$	18:2	35.2	31.9	33.1	30.1
Linolenic acid	$C_{18}H_{32}O_2$	18:3	0.3	0.3	0.2	0.3

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Table 4 – The comparison of physico-chemical properties of *Jatropha curcas* crude oil and methyl esters.

Properties	IIITJ8		IITJ9		IITJ18		IITJ20		ASTM D6751	ASTM testing method
	OIL	FAME	OIL	FAME	OIL	FAME	OIL	FAME		
Specific gravity	0.86	0.85	0.86	0.83	0.86	0.83	0.86	0.84	0.860–0.900	ASTM D854-10
Kinematic viscosity at 40 °C (cst)	77.40	6.70	46.60	6.30	57.70	6.10	53.70	6.10	1.9–6	ASTM D445
Flash point (°C)	310	185	309	185	310	185	308	185	130	ASTM D92
Fire point (°C)	330	190	330	190	330	190	330	190	–	ASTM D92
Refractive index (27 °C)	1.46	1.45	1.46	1.45	1.46	1.45	1.46	1.45	–	–
Moisture content (%)	0.31	0.23	0.35	0.25	0.39	0.23	0.22	0.20	0.50 max	ASTM D2216-10
Cloud point	11	8	10	8	11	8	11	8	Not specified	ASTM D2500
Pour point	4	2	4	2	4	2	4	2	−15 to 10	ASTM D2500
Calorific value (MJ/Kg)	39.80	41.09	39.23	40.27	39.73	40.85	38.31	39.56	–	ASTM D2015-85
Acid value (mg KOH g^{-1})	2.24	0.44	2.56	0.44	2.82	0.43	2.90	0.43	0.50max	AOCS (Te 1a-64)
FFA content	1.12	0.221	1.28	0.221	1.41	0.218	1.45	0.218	0.25 max	AOCS (Te 1a-64)
Iodine value	87.56	88.83	111.03	112.30	102.15	100.25	95.17	94.54	120 max	AOCS (Tg 1–64)

of standard ASTM specifications. Similarly, viscosity of oils samples was measured using a standard protocol. Viscosity of the oil increases with increase in molecular weight and decreases with increase in unsaturation level and temperature [19]. The kinematic viscosity of the oil at (40 °C) was found to be 77.40, 46.60, 57.70 and 53.70 cst respectively, which is much higher as compared to conventional diesel (Table 4). These high viscosity oils cannot be used directly in engine. High viscosity causes injector fouling and other engine operational problems. Therefore, before application in diesel engine, processing is required to reduce the viscosity of the oil. The flash point of the oil samples under study was found to be 310, 309, 310 and 308 °C respectively (Table 4). The result shows that the flash point of oil samples is much higher as compared to conventional diesel. Similarly, the fire point of oil was found at 330 °C which is much higher than diesel. Refractive index is the degree of the deflection of a beam light that occurs when it passes from one medium to the other. The refractive index value increases with the degree of unsaturation. Refractive index of four accessions was found to be 1.46. This value indicates the presence of long chain unsaturated fatty acids in Jatropha oil.

Moisture content of oils was determined using Karl fisher titrator. Moisture content is a qualitative parameter of oil, which influences the storage life of fuel. High moisture content may serve as a medium for microbial growth. Microbial growth in the oil may leads to damage of tank and emulsion formation [20]. Besides this, it initiates oxidation of oil which effects longetivity of engines and reduced shelf life of the oil. The moisture content of the oil was found to be 0.31, 0.35, 0.39 and 0.22% respectively, which are within acceptable range of standard values. The flow characteristic of oil was observed under low temperature. The result shows that the cloud point of IITJ8, IITJ18, IITJ20 was observed to be 11 °C and for IITJ9, it was at 10 °C. The pour point of 4 °C was observed for all the four genotypes. The observed cloud point and pour point values of Jatropha oil are much higher as compared to conventional diesel. The calorific value for the oil was measured in an oxygen bomb colorimeter (Table 4). The data obtained from experiment for all four accession showed high calorific value in the range of 39.23–39.80 MJ/Kg (Table 4). The acid value of the oil determines the process of transesterfication i.e. either one step or two step process [21]. The acid value of the IITJ8, IITJ9, IITJ18 and IITJ20 was measured to check the free fatty acid content in the oil sample, and it was found to be 2.24, 2.56, 2.82 and 2.90 mg/KOH respectively (Table 4). The acid values for all the accessions were found to be quite less compare to the reported acid values of *J. curcas* from various other places [21–23]. These differences in acid value can be related with the wild origin, maturation stage of seeds and different storage condition [22]. As per the values reported in the literature FFA content of Jatropha oil varies in the range of 5–15% which is far beyond the capacity of conversion to biodiesel via single step alkali catalyzed transesterification. But in the present study FFA content of oil was found to be very less. So, single step alkali catalyzed reaction was performed for conversion of oil in to biodiesel. This single step transesterification yields substantially higher conversion rate and decreased the reagent use and reaction time as compared to two step transesterification process. The high FFA content increases the formation of fatty acids salts (soap) and conversion rate decreased which cause problem in separation of glycerol at washing step. Iodine value measures the unsaturation content in the oil sample. Iodine values of the oil were found to be 87.56, 111.03, 102.15 and 95.17 g/100 g respectively (Table 4).

3.4. *Characterization and evaluation of synthesized methyl esters*

The fuel properties of methyl esters of four high oil yielding accession were determined using the standard protocol and presented in Table 4. During the study it was observed that the specific gravity which influences the fuel atomization [21] was reduced after methanolysis. The obtained values for methyl esters of four accessions are within the acceptable range of ASTM standards (Table 4). As described above the viscosity which is the major problem in the oil samples for engine operation was substantially decreased after transesterification. The decreased value of the viscosity at (40 °C) was found to be 6.70, 6.30, 6.10 and 6.10 cst respectively. The values are almost within the acceptable range of ASTM standards (Table 4). Similarly, flash and fire point values were also found to be reduced after transesterification and the obtained

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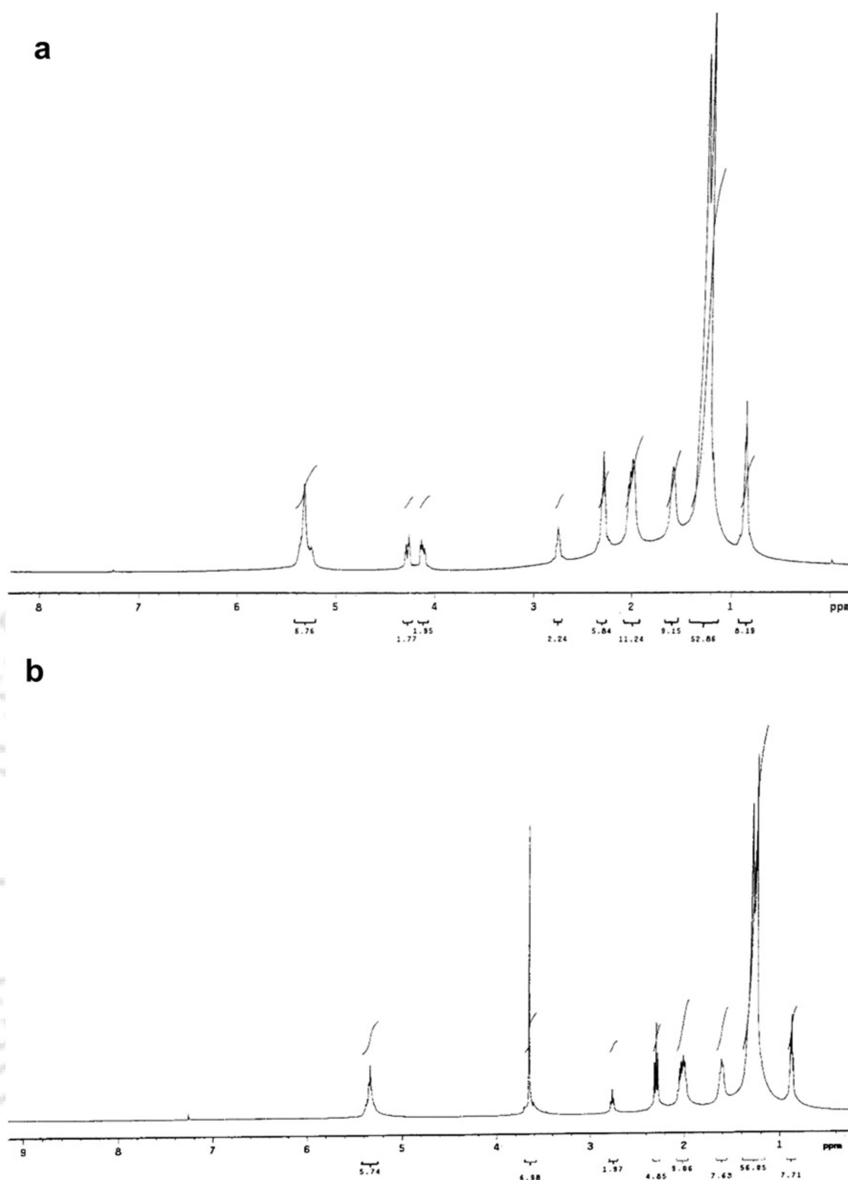

Fig. 2 – ^{1}H NMR spectrum of *Jatropha curcas* oil and methyl ester. a, ^{1}H NMR spectrum of crude oil of IITJ8 accession. b, ^{1}H NMR spectrum of methyl ester of IITJ8 accession, the strong singlet peak at 3.659 ppm is indicative of due to oxymethylic hydrogen is indicative of methyl esters.

values are in the range of 185 and 190 °C respectively. Since, flash point and fire point values are depends on viscosity, therefore, decrease in the viscosity values after trans-esterification might be one of the cause for reduction. Iodine value of the prepared methyl esters showed almost similar value as that of the oil, that is because this parameter solely dependent on the origin of the oil [24]. Acid value is another measure of qualitative character of biodiesel. As per the ASTM standard, acid value of transesterified product should not be more than 0.5 mg KOH/g. The acid values of the methyl esters of the four oil samples under the study was found to be 0.44, 0.44, 0.43 and 0.43 mg KOH/g respectively, which is below the maximum limits of the standards (Table 4). Similarly, the moisture content of the prepared methyl esters samples was determined using Karl fisher titrator. After transesterification the moisture content of IITJ8, IITJ9, IITJ18 and IITJJ20 methyl esters was found to be 0.23, 0.25, 0.23, and 0.20% respectively, which is well below the maximum acceptable limits. Cold flow properties such as cloud point and pour point of methyl ester was found to be improved after transesterification. Cloud point and pour point of methyl esters was measured as 8 °C and 2 °C respectively. But these values are still much higher than the conventional diesel. The heating value is one of the essential properties for evaluation of biodiesel, which provides the suitability of fuels as alternative to diesel fuels [25]. Calorific value for IITJ8, IITJ9, IITJ18 and IITJ20 methyl esters was obtained as 41.09, 40.27, 40.85 and 39.56 MJ/Kg respectively. The result shows that calorific value of methyl esters is higher than the corresponding oils (Table 4). All these properties combined together have shown that, IITJ8, IITJ9,

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IITJ18 and IITJ20 could act as the potential candidates for biodiesel production in North East India.

3.5. ^{1}H NMR spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy was employed to monitor the transesterification reaction. The use of ^{1}H NMR for monitoring the yield of transesterification reaction was first proposed by Glebard et al., (1995) [26]. In case of ^{1}H NMR spectra of methyl esters, signal appears in the region δ 3.7 ppm which indicates the presence methylic esters group [27].

The characteristic peak of methoxy protons was observed as a singlet at δ 3.65 ppm and this signal was attributed to methyl esters, which was absent in the oil. Fig. 2a and b, represent the ^{1}H NMR spectrum of IITJ8 oil and IITJ8 methyl esters respectively. In case of ^{1}H NMR spectra of IITJ8 oil, multiplet peaks were observed in the region δ 4.11–4.115, δ 4.266–4.306 ppm and δ 5.30–5.34 ppm, due to oxymethylic hydrogen that are characteristic of triglycerides. Fig. 2b shows the ^{1}H NMR spectrum of IITJ8 methyl esters, the strong singlet peak at 3.659 ppm is indicative of conversion of parent oil to methyl esters. So, from the NMR spectrum of oil and methyl esters, it could be verified that Jatropha oil conversion into biodiesel was successfully completed.

3.6. Thermal stability of Jatropha oil and methyl esters

Thermal stability of Jatropha oil and its methyl esters was determined from onset temperature of thermal decomposition under nitrogen atmosphere. TGA curve of four oil sample under nitrogen atmosphere is shown in Fig. 3. The curve shows three consecutive stages of thermal decomposition of the oil samples. Fig. 3a depicts the TGA curve of IITJ8, IITJ9, IITJ18 and IITJ20 oil samples. From the figure it can be seen that IITJ8, IITJ9 and IITJ18 show similar decomposition pattern. The first phase of decomposition start at 310–315 °C and second phase extended up to 470 °C which leads to rapid weight loss. The final stage of decomposition, where pyrolyzed product of second phase fully decomposed extended from 470 to 900 °C. But the TGA curve of IITJ20 showed decomposition at little lower temperature than the other three accessions. In the first stage evaporation starts at 240–280 °C, extended up to 540 °C where rapid weight loss was occurred. Final decomposition occurred between 540 and 900 °C. The difference in the decomposition pattern may be due to the presence of less moisture in the IITJ20 oil sample (0.22%) as compared to other three samples. TGA analysis of methyl esters samples was carried out under similar condition. TGA curve of methyl esters of IITJ8, IITJ9, IITJ18 and IITJ20 samples represent the three stage decomposition at 120–125 °C, 125–360 °C and 360–900 °C respectively (Fig. 3b).

From the TGA curves of the oil samples and methyl esters it was observed that, the process of degradation of Jatropha methyl esters initiates and completed within a temperature range inferior to the respective oil samples. Molecular tension produced by bulky triglycerides molecule in the oil sample which could be the reason for thermal stability of oil [28]. Besides this, high viscosity might be the reason for slow degradation process [15].

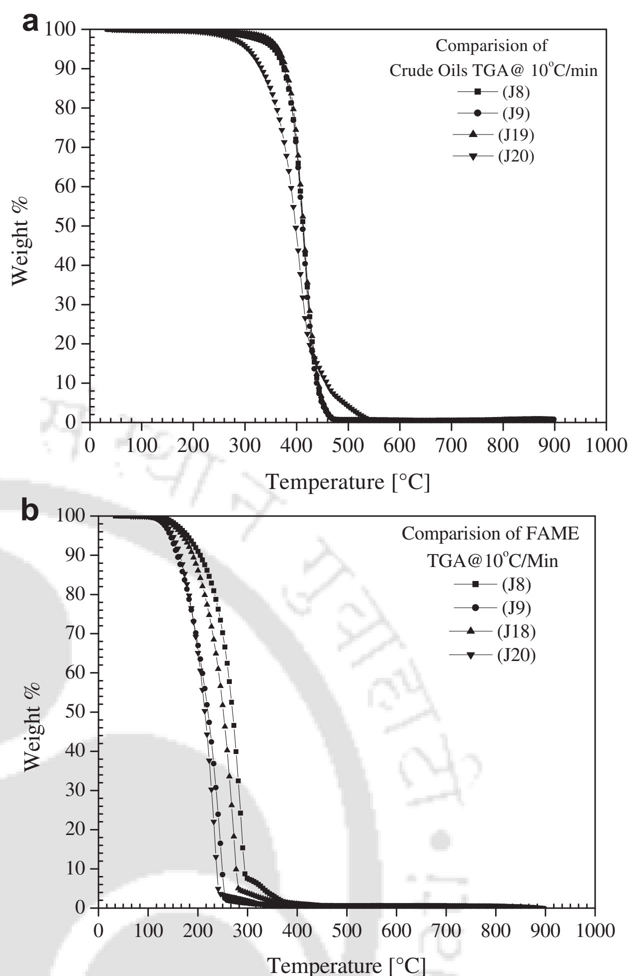

Fig. 3 – TGA profile of *Jatropha curcas* crude oil and methyl esters under nitrogen environment. (a) Comparison of TGA profile of IITJ8, IITJ9, IITJ18 and IITJ20 crude oil. (b) Comparison of TGA profile of IITJ8, IITJ9, IITJ18 and IITJ20 methyl esters.

The poor volatility and high viscosity of the oils are the major challenges to run modern diesel engines with plant oils. The onset temperature for volatilization and distillation was calculated from respective TGA curves of Jatropha oil and methyl esters and presented in Table 5. The result shows that onset temperature of thermal degradation of methyl esters was lower as compared to oil samples.

In case of IITJ8 oil sample weight loss was negligible below 300 °C. But after that rapid degradation was observed at 310 °C compared to its methyl esters at 120 °C. During this study it was observed that for all the oil samples 50% weight loss was occurred at around 420 °C, while in case of methyl esters it was around at 280 °C. All the volatile components of the oil which accounted for almost 90% weight were decomposed at around 440 °C, whereas in case of respective methyl ester 90% weight loss was observed at 295 °C. The remaining 10% was pyrolysis product which is highly viscous liquids, under goes secondary decomposition. The temperature for secondary decomposition extended up to 470–530 °C for oil and 320–380 °C for methyl esters. The residue was completely

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Table 5 – Volatilization and distillation temperature obtained from TGA profile of crude oil and methyl esters.

Sample name	Onset temperature for volatilization (°C)		Distillation temperature (°C)					
			10%		50%		90%	
	Crude oil	FAME	Crude oil	FAME	Crude oil	FAME	Crude oil	FAME
IITJ8	310	120	390	210	420	280	440	295
IITJ9	310	120	400	160	420	225	450	260
IITJ18	310	120	408	200	425	260	440	280
IITJ20	240	120	350	175	410	220	465	245

burnt out after heating up to 900 °C for both oil and methyl esters. The data in the Table 5 confirms that oil is more thermally stable and less volatile as compared to methyl esters. Further it is also confirmed that methyl esters shows close proximity with conventional diesel.

3.7. *Oxidative stability of Jatropha oil and methyl esters*

Oxidative stability is the quality indicative parameter for methyl esters. It is defined as the resistance of the oil against oxidation. The consequence of lipid oxidation results in decreasing the shelf life of the oil. The oxidation of long chain methyl esters initially results in accumulation of hydro peroxides. Then gradually it polymerizes forming insoluble sediments that plugged filters, fouled injectors and interfered with engine operation [15]. To measure the oxidative stability of the oil as well as methyl esters TGA analysis was performed in oxygen atmosphere under same conditions (Fig. 4). Fig. 4a shows TGA profile for IITJ8, IITJ9, IITJ18 and IITJ20 oil samples under oxygen environment, whereas Fig. 4b shows the oxidative degradation profile of their respective methyl esters. During the analysis it was observed that the onset temperature of oxidative degradation for oil samples was in the range of 230–245 °C, whereas, in case of methyl esters it was in the range of 115–120 °C (Table 6). That is mainly because methyl esters are less viscous than oil. This low viscosity increases the contact between oxygen and ester molecules resulting higher oxygen diffusion [29]. The vegetable oil contains naturally occurring antioxidants such as tocopherols, sterols and tocotrienols, but the purification process destroys these natural antioxidants and hence becomes prone to oxidation [30]. The oxidative stability of methyl ester can be improved either by using synthetic antioxidants which are available in market or vegetable oil based antioxidant additives. It is observed that blending Jatropha biodiesel with palm biodiesel improves oxidative stability [31]. So, further research and development on Jatropha based biodiesel will make it more attractive to replace fossil fuels.

Table 6 – Onset temperature for oxidative degradation for *Jatropha curcas* oil and methyl esters.

Sample name	Onset temperature for oxidative degradation (°C)	
	Crude oil	FAME
IITJ8	230	115
IITJ9	240	115
IITJ18	245	120
IITJ20	240	115

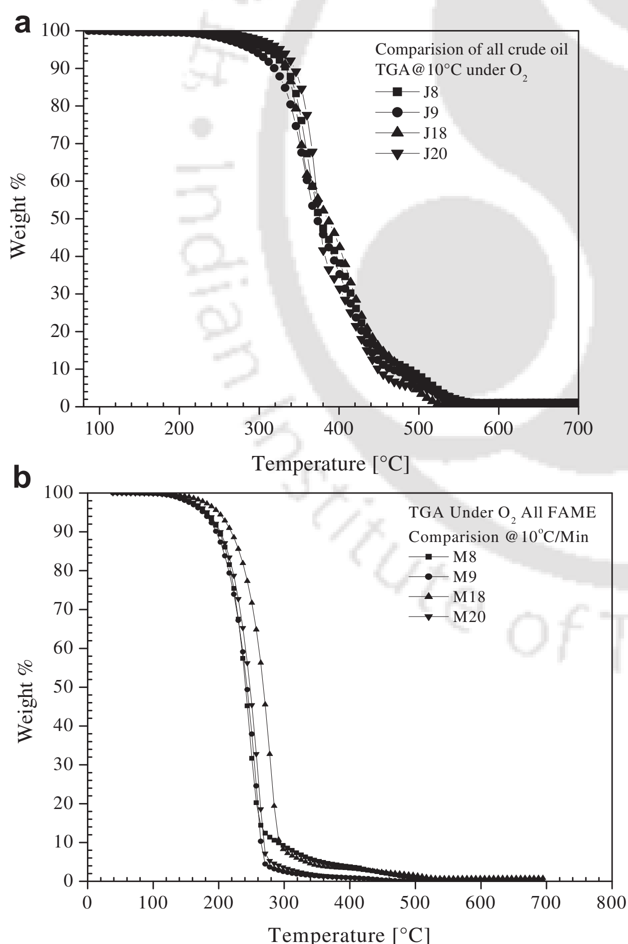

Fig. 4 – TGA profile of *Jatropha curcas* crude oil and methyl esters under oxygen environment. (a) Comparison of oxidative stability of IITJ8, IITJ9, IITJ18 and IITJ20 crude oil. (b) Comparison of oxidative stability of IITJ8, IITJ9, IITJ18 and IITJ20 methyl esters.

4. Conclusions

Current investigation on the oil content and fuel properties of *J. curcas* provides valuable information on potential resources for biodiesel production in North East India. Physicochemical characterization of oil and methyl esters established the

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suitability of the biodiesel to use in diesel engine. The four accessions used in this study showed low level of FFA, therefore single step alkali catalyzed transesterification was found to be sufficient for biodiesel production. From the study it can be concluded that these four accessions can be used for large scale propagation and cost-effective biodiesel production in North East India. However, more extensive and experimental study needs to be carried out to investigate combustion, emission characteristics and its performance on DI-CI Engine. Therefore, we still need to focus on the process design, and kinetics of Jatropha oil transesterification in a batch reactor and analysis in biodiesel- fueled engine to establish Jatropha biodiesel as successful alternative fuel.

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Research article

Excess copper induced oxidative stress and response of antioxidants in rice

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ABSTRACT

To investigate the effects of copper (Cu), rice plant (*Oryza sativa*. L. var. MSE-9) was treated with different Cu concentrations (0, 10, 50 and 100 μM) for 5 days in hydroponic condition. Gradual decrease in shoot and root growth was observed with the increase of Cu concentration and duration of treatment where maximum inhibition was recorded in root growth. Cu was readily absorbed by the plant though the maximum accumulation was found in root than shoot. Hydrogen peroxide (H_2O_2) production and lipid peroxidation were found increased with the elevated Cu concentration indicating excess Cu induced oxidative stress. Antioxidant enzymes superoxide dismutase (SOD), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) and glutathione reductase (GR) were effectively generated at the elevated concentrations of Cu though catalase (CAT) did not show significant variation with respect to control. Ascorbate (ASH), glutathione (GSH) and proline contents were also increased in all the Cu treated plants compared with the control. SOD isoenzyme was greatly affected by higher concentration of Cu and it was consistent with the changes of the activity assayed in solution. The present study confirmed that excess Cu inhibits growth, induced oxidative stress by inducing ROS formation while the stimulated anti-oxidative system appears adaptive response of rice plant against Cu induced oxidative stress. Moreover proline accumulation in Cu stress plant seems to provide additional defense against the oxidative stress.
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1. Introduction

Heavy metal toxicity is one of the major environmental problems to the present world because of its increasing level caused by both natural sources (e.g., wind-blown dust, decaying vegetation, forest fires and sea spray) and human activities (e.g., mining, metal production; wood production and phosphate fertilizer). Even in trace concentration, they caused serious problem to all the organisms. Among the heavy metals, copper (Cu) is an essential micronutrient for plant growth and various biochemical processes. It is required in much important biological function since they are constituents of many enzymes and proteins. It is a co-factor of enzymes like plastocyanin, cytochrome c, and Cu/Zn^{-} superoxide dismutase (Cu/Zn–SO). However, excessive Cu adversely affects plant growth and metabolism. Even slightly higher concentration of Cu from the optimal level induces toxicity to the plant [1]. Being a redox active metal, Cu catalyzes the production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•), via Haber–Weiss and Fenton reactions [2]. ROS are damaging to essential cellular components such as DNA, proteins and lipids therefore induction of ROS production led to oxidative stress affecting plant growth and alteration of antioxidant system [3].

Plants have evolved certain mechanisms to tolerate heavy metal stress such as metal exclusion, metal accumulation and binding of heavy metal by strong ligands like cysteine-rich proteins, metallothioneins (MTs) and thiol-rich peptides, phytochelatins (PCs) [4,5]. Moreover, plants have stimulated antioxidant system to combat the oxidative injury induced by the heavy metals [6]. These include several ROS-removing enzymes such as superoxide dismutase (SOD), catalases (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), and low molecular mass antioxidants scavengers such as ascorbate (ASC) and glutathione (GSH). It has been suggested that Cu induced antioxidative reactions in the roots of *Brassica juncea* and in Arabidopsis [4]. These induced cellular antioxidants scavenged ROS thereby preventing the damage caused by the overproduction of ROS. Generation of proline is also one of the vital responses of plant under Cu toxicity which is possibly associated with the protection

Abbreviations: APX, ascorbate peroxidase; ASH, ascorbate; CAT, catalase; GPX, guaiacol peroxidase; GSH, glutathione; GR, glutathione reductase; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; H_2O_2, hydrogen peroxide.

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of plant cells against oxidative damage and with signal transduction [7]. Proline might protect plants from metal toxicity by chelating heavy metals in the cytoplasm or as a hydroxyl radical scavenger [8]. It can be considered as a non-enzymic antioxidant which is involved in counteracting the ROS damage. However different plants, plant parts and metals evolved different strategies to resist the toxicity.

Rice is an important staple food of the world population containing high nutritional quality among cereal crops. Half of the world population depends on rice for survival but due to certain heavy metal toxicity including Cu caused reduction of the crop yield. The present investigation was undertaken to test the effects of Cu on growth, induction of oxidative stress and antioxidative responses in rice (*Oryza sativa* L.) variety MSE-9.

2. Results

2.1. Changes of growth and copper uptake

The effect of different Cu concentrations on the growth of rice plant was given in Table 1. A gradual decrease in shoot and root elongation was observed with the increase in Cu concentration and length of treatment. Maximum inhibition was occurred at 100 μM Cu after 5th day in shoot (18.84%) and root (27.59%) with respect to control. At the same concentration, fresh mass of shoot is decreased by 35.31% while in root 44.44% is found to be decreased (Table 1) suggesting higher impact of Cu toxicity in root than shoot. From the result, it also seen that biomass is more susceptible to Cu toxicity than length. Cu content in root and shoot (Table 1) increased gradually with increasing Cu concentrations in the treatment though root showed greater Cu content than shoot.

2.2. Hydrogen peroxide level

The level of H_2O_2 increased progressively both in shoot and root with the increase of Cu concentrations and duration of treatment (Fig. 1A, B). Maximum increase was observed at the highest concentration (100 μM) after 5th day where shoot showed 32.82% increment in comparison with control. Much higher levels were observed in root with 44.07% and 50.11% increased over the control exposed to 100 μM Cu after 1st day and 5th day respectively. The H_2O_2 production was quite proportional with concentration and duration of Cu treatment.

2.3. Lipid peroxidation

Lipid peroxidation measured in terms of MDA increased in all the stressed plants comparing with control. MDA content in shoot and root gradually increased with the increase of Cu concentrations (Fig. 1C, D) and its content was more in root than that of shoot in all the experimental condition. Significant increase of MDA content was found at 100 μM Cu in root after 5th day of treatment (43.82% over control). The increase in MDA content under Cu treatment can be interrelated with an increase in concentrations and duration of treatment.

2.4. Ascorbate and glutathione contents

Treatment of the rice plant with different concentrations of Cu increases ASH content in both shoot and root (Table 1). The ASH contents increased by 24.13% in shoot while 29.31% in root at 100 μM after 5th day of treatment over control. GSH content was also enhanced in shoot and root of 10, 50 and 100 μM of Cu with respect to control (Table 1). Maximum increase was observed at 100 μM Cu after 5th day of treatment by 44.20% in shoot while 54.25% in root.

2.5. Proline content

As shown in Table 1, proline content increased with the increasing Cu concentrations in the treatment. The proline content increased by 26.89%, 45.58% and 67.60% in shoot while 19.12%, 54.81% and 110.43% in root at 10, 50 and 100 μM Cu respectively with respect to control after 1st day of treatment. Almost similar

Table 1
Effect of different concentrations of Cu on growth, fresh mass, contents of Cu, ASH, GSH and proline in shoots and roots of rice plant after 1st day and 5th day of treatment. The data presented are mean of three separate experiments ± SE. * indicates significant mean difference from control at $P = 0.05$ according to LSD test.

Parameter		0 μM	10 μM	50 μM	100 μM
Shoot					
Growth (cm)	1st day	14.65 ± 0.579	13.58 ± 0.630	13.10 ± 0.826	12.93 ± 0.866
	5th day	16.88 ± 0.781	16.12 ± 0.758	14.60 ± 0.720	13.70 ± 0.917
Fresh mass (g)	1st day	0.266 ± 0.033	0.235 ± 0.038	0.194 ± 0.038	0.184 ± 0.038
	5th day	0.354 ± 0.029	0.324 ± 0.030	0.317 ± 0.029	0.229 ± 0.032*
Cu [$\mu g\, g^{-1}$ (f.m.)]	1st day	0.049 ± 0.007	0.098 ± 0.005	0.125 ± 0.008	0.298 ± 0.008*
	5th day	0.044 ± 0.012	1.812 ± 0.317	3.660 ± 0.234*	4.874 ± 0.265*
Ascorbate [$\mu mol\, g^{-1}$ (f.m.)]	1st day	569.40 ± 61.97	588.87 ± 40.19	613.65 ± 59.70	673.63 ± 61.26
	5th day	573.97 ± 58.54	596.37 ± 52.14	639.98 ± 57.26	712.45 ± 64.72
Glutathione [$\mu mol\, g^{-1}$ (f.m.)]	1st day	2537.45 ± 217.25	2733.74 ± 228.16	2591.07 ± 222.69	3725.01 ± 224.11*
	5th day	2855.58 ± 192.97	2907.16 ± 193.29	3445.10 ± 204.12	4117.92 ± 190.69*
Proline [$\mu mol\, g^{-1}$ (f.m.)]	1st day	2.034 ± 0.238	2.581 ± 0.301	2.961 ± 0.320	3.409 ± 0.300*
	5th day	2.283 ± 0.339	3.102 ± 0.361	2.932 ± 0.363	4.542 ± 0.278*
Root					
Growth (cm)	1st day	10.05 ± 0.335	10.06 ± 0.330	8.95 ± 0.384	8.27 ± 0.524
	5th day	12.47 ± 0.398	12.63 ± 0.369	10.18 ± 0.497	9.03 ± 0.471*
Fresh mass (g)	1st day	0.021 ± 0.0031	0.021 ± 0.0033	0.018 ± 0.004	0.017 ± 0.0034
	5th day	0.036 ± 0.0033	0.037 ± 0.032	0.025 ± 0.0032*	0.020 ± 0.0036*
Cu [$\mu g\, g^{-1}$ (f.m.)]	1st day	0.090 ± 0.022	0.384 ± 0.066	1.522 ± 0.106*	4.863 ± 0.104*
	5th day	0.106 ± 0.053	14.60 ± 0.505*	24.30 ± 0.714*	38.56 ± 0.688*
Ascorbate [$\mu mol\, g^{-1}$ (f.m.)]	1st day	583.79 ± 53.98	577.04 ± 53.36	615.66 ± 50.02	679.45 ± 46.22
	5th day	539.80 ± 50.86	592.81 ± 52.05	646.30 ± 53.30	698 ± 51.16
Glutathione [$\mu mol\, g^{-1}$ (f.m.)]	1st day	3686.68 ± 192.69	4629.29 ± 203.54*	5095.24 ± 199.84*	5226.48 ± 224.37*
	5th day	3889.27 ± 208.74	4951.57 ± 198.19*	5654.88 ± 213.81*	5999.17 ± 216.81*
Proline [$\mu mol\, g^{-1}$ (f.m.)]	1st day	2.233 ± 0.345	2.660 ± 0.420	3.457 ± 0.528	4.699 ± 0.321*
	5th day	2.619 ± 0.358	2.860 ± 0.420	3.854 ± 0.402	5.408 ± 0.548*

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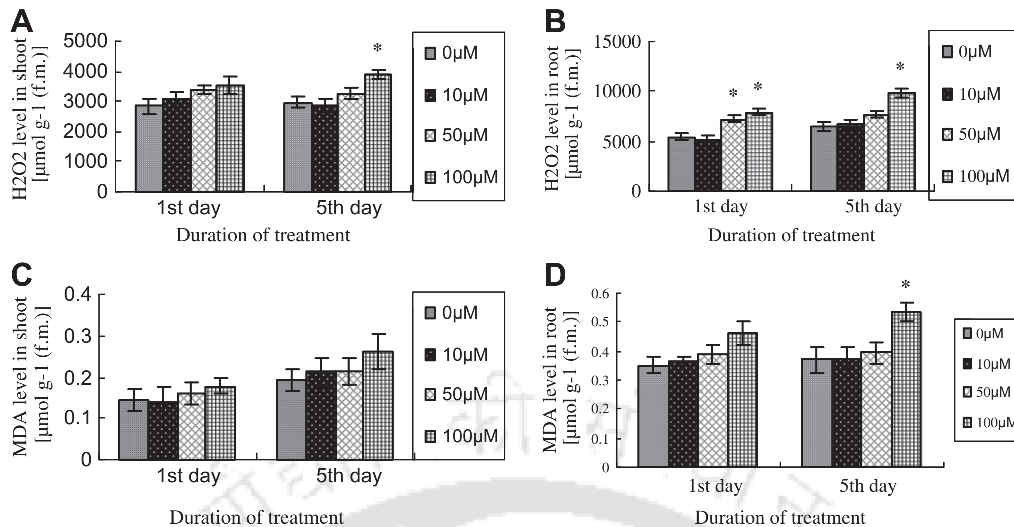

Fig. 1. Effect of different concentrations of Cu on contents of MDA in shoot (A), root (B) and H_2O_2 in shoot (C) and root (D). Data presented are mean ± SE ($n = 3$). *Significant mean difference from control at $P = 0.05$ in multiple comparison by LSD test.

pattern of enhancement in the proline content was recorded after 5th day where maximum content was recorded at 100 µM Cu by 98.95% in shoot and 106.49% in root over control.

2.6. Antioxidant enzymes activities

The antioxidant enzymes are important components in preventing oxidative stress in plants by scavenging free radicals and peroxides with the elevation of their activities when exposed to stressful conditions. Almost all the antioxidant enzymes have been greatly activated under stress condition.

In the present study, it is found that SOD activity increases by 62.85% and 64.58% at 100 µM Cu with respect to control in shoot and root respectively after 1st day recorded exposed to 100 µM Cu. Results of the isoenzyme patterns suggested that four isoenzyme bands (i, ii, iii and iv) were detected in the shoot (Fig. 2B) where band iv showed moderate expression at 10 µM Cu as compared to the expression under higher Cu concentrations. In root, three isoenzyme bands (i, ii and iii) were found and SOD iii was detected only at the higher concentration of Cu, 50 µM and 100 µM (Fig. 2D). Results showed the levels of SOD induced in response to copper stress however they differ in different copper concentration.

GPX activity has been increased in both shoot and root with an increase in Cu levels and duration of treatment (Fig. 3A, B). After 5th day of treatment, higher activity of GPX was observed where 19.52%, 33.81% and 68.49% in shoot and 22.83%, 43.47% and 80.46% in root increased respectively at 10, 50 and 100 µM Cu comparing to the control. The activity was higher in root than shoot. GR activity was increased in all the treated plant where significant increase was observed at the higher concentration of Cu after 5th day of treatment. Treatment with 100 µM increased the GR activity by 47.74% in shoot (Fig. 3C) and by 66.04% in root (Fig. 3D) over control. In this enzyme activity also root showed greater activity than shoot. The activity of APX enzyme increased progressively in a concentration and time dependent manner in both shoot and root. The activity of this enzyme was dramatically increased at 50 and 100 µM Cu after 5th day of treatment with 42.77% and 69.26% in shoot (Fig. 3E) and 48.44% and 69.26% in root (Fig. 3F) over the control respectively. In this study, CAT activity of the treated plants was almost equal with the control in both shoot and root in all the experimental conditions.

3. Discussion

3.1. Changes of growth and copper uptake

The decrease in growth of rice plant when raised under increasing level of Cu suggested Cu induced toxicity at elevated concentration and the impact was found more in root than shoot. The greater sensitivity of root to Cu toxicity might be due to the higher Cu content in root as maximum accumulation of Cu in rice root was observed. Similar observation was reported in *Elsholtzia splendens* [9] under Cu treatment. On the basis of this result, Cu toxicity is directly correlated with the accumulation of Cu in the plant. Greater Cu content in root than shoot indicates adoption of exclusion mechanism to tolerate the toxicity in which the roots accumulate the metals preventing its subsequent transport to the shoots [3]. Our results reconfirmed the earlier report showing correlation of Cu tolerance and its greater accumulation in root with poor translocation to shoot in Cu-tolerant plants [10]. A slight increase in Cu concentration from the optimal requirement caused toxicity to most of the plants. Even 4 µM Cu drastically inhibited the root length of *Paspalum distichum* and *Cynodon dactylon* [11]. However, in our study not much significant changes in root and shoot length and biomass were recorded even at 50 µM Cu concentrations during early stages of Cu stress suggesting the Cu tolerance of MSE-9 rice variety under study.

3.2. Hydrogen peroxide level

In the current finding increase in Cu concentration led to the increase in H_2O_2 production. The root showed higher H_2O_2 content than shoot indicating root is the primary site for ROS production. It might be due to the higher Cu content in root as Cu induced ROS production in cells. Copper and other heavy metals like Cr, Zn and Ni have been reported previously to produce ROS like $O_2^{\bullet -}$, H_2O_2 and OH• which in turn induce oxidative damage and cell death in plants [12–14].

3.3. Lipid peroxidation

As MDA is a product of lipid peroxidation, the elevation in MDA content clearly reflects cell wall damage. Our results indicate that excess Cu increases oxidative stress as is evident from increased

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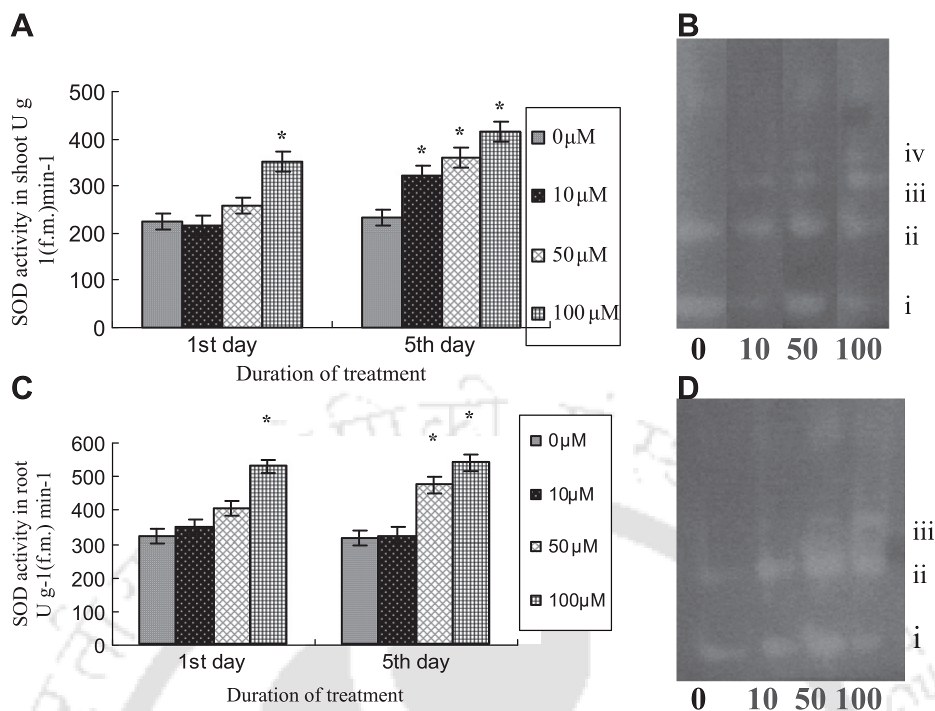

Fig. 2. Effect of Cu on the activities and isoenzyme patterns of SOD in shoot (A and B) and root (C and D). B and D showed the isoenzyme activity in shoot and root respectively after 5th day of treatment under different concentrations of Cu, 0 μM, 10 μM, 50 μM and 100 μM. Data presented are mean ± SE ($n = 3$). *Significant mean differences from control at $P = 0.05$ in multiple comparison by LSD test.

lipid peroxidation. It is accordance with the previous findings [15] that MDA accumulated greatly after the exposure of Cu and cell membrane is the primary site of Cu toxicity. It might be due to the overproduction of ROS under Cu stress which is highly destructive to cell membrane.

3.4. Ascorbate and glutathione contents

The present study showed that ASH and GSH contents increase in all the concentrations of Cu of this experiment. Significant increase in GSH content in all the treated plant in root reveals increase in Cu tolerance as GSH is involved in keeping the ROS in balance. Greater enhancement in GSH content in root might be due to combat the damage induced by the overproduction of H_2O_2 in root. Pastori and Foyer [16] reported that GSH, as an antioxidant, helped to withstand oxidative stress in transgenic lines of tobacco. Increased in the GSH content in all the Cu treated plant also reflects biosynthesis of phytochelatins (PCs), as GSH is a precursor for PCs [17] which may involve in detoxification and tolerance by chelating the metals [5]. ASH is also one of the main antioxidant that contributes to resistance against oxidative stress. It influences many enzyme activities and minimizes the damage caused by oxidative process through synergic function with other antioxidants [18]. Increment in ASH content under Cu stress indicates its involvement in the Cu tolerance mechanism.

3.5. Proline content

The result suggested that accumulation of proline responses to Cu and it varies with respect to the toxicity caused by Cu treatment. Accumulation of proline in tissue/organs of plants under heavy metal pollution has been reviewed by Alia and Pardha Saradhi [19]. Proline accumulation prevents membrane distortion and acts as a hydroxyl radical scavenger. Mehta and Gaur [20] also noted a protective role of proline in mitigating metal-induced lipid peroxidation in *Chlorella vulgaris*. Thus greater accumulation of proline under high Cu level of the present study suggested the protective role of proline to the plant to survive Cu stress.

3.6. Antioxidant enzymes activities

SOD constitutes the first line of defense against ROS. SOD is a metalloenzyme that dismutate superoxide radical to H_2O_2 and oxygen. Increase in SOD activities in stressed plants was indicative of enhanced $O_2^{\bullet-}$ production and oxidative stress tolerance and has been proposed as an important enzyme for plant stress tolerance. The results of the current study showed constant detoxification of the $O_2^{\bullet-}$ that might have been generated. Recent report also showed increase in SOD activity under Cu stress in *Elsholtzia haichowensis* [21].

In this study, GPX appeared to be an important enzyme in overcoming Cu imposed oxidative stress as there has been a considerable increase in GPX activities in the stressed plants. This was in accordance with the previous report in *Pavlova viridis* [22]. GPX is the antioxidant enzyme capable of removing H_2O_2, neutralizing or scavenging free radicals. Significant increase in GPX activity in the present study reflects its vital role in scavenging H_2O_2 produced by the Cu stress.

Increased GR activity in the Cu treated plant could be an acclimatization step against Cu stress as it is an important enzyme essential for the appropriate functioning of the antioxidant system in plants. It involved in the enzymatic detoxification of ROS and contributes to the maintenance of a higher GSH to GSSG ratio. Similar finding has been reported in *B. juncea* under Cd stress [23].

Increase in APX activities both in shoot and root of the rice plant when were exposed to Cu stress showed its role in the adaptation process against Cu induced oxidative stress. Enhancement of APX activity under Cu stress has been previously reported [24]. APX

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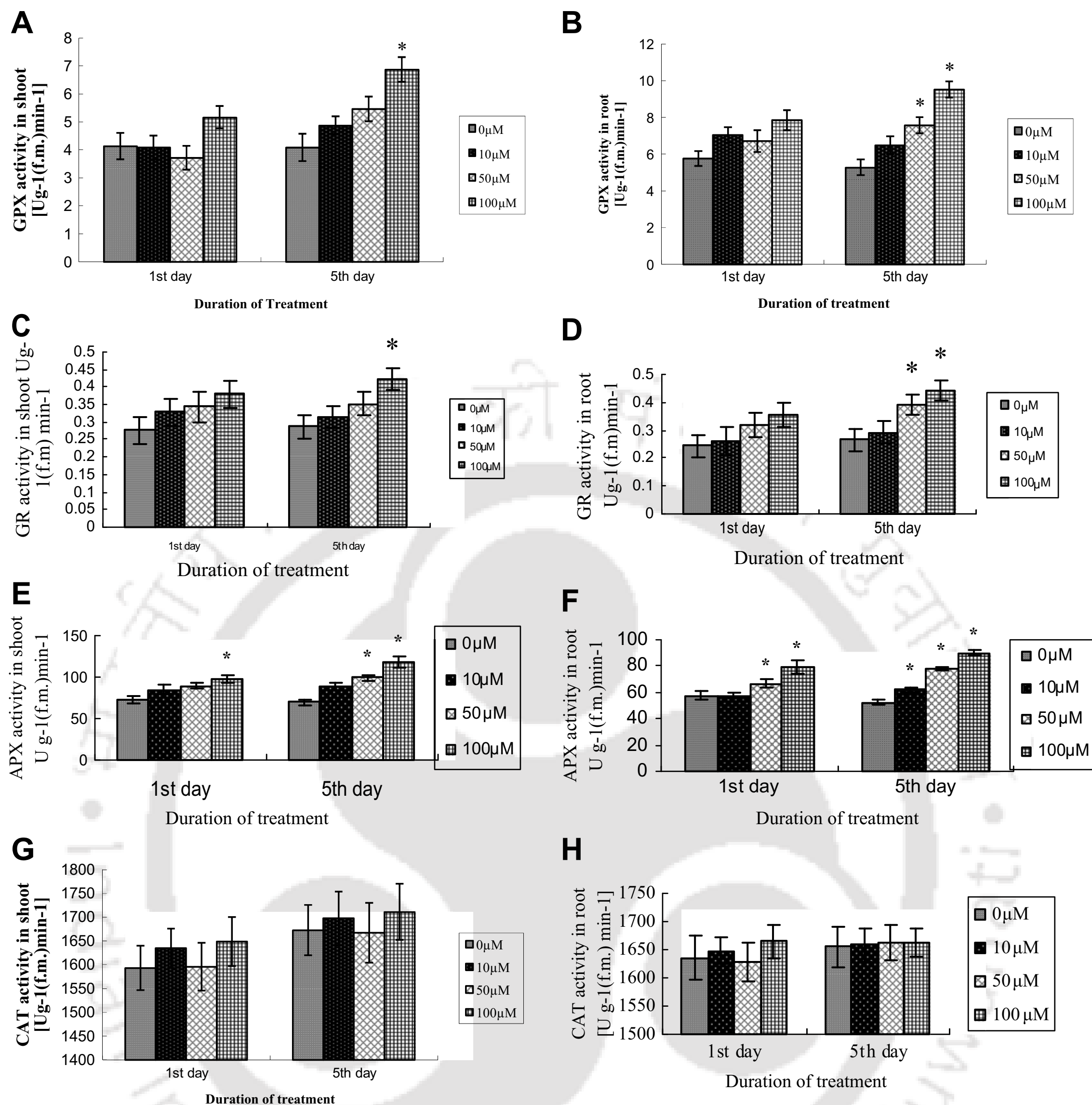

Fig. 3. Effect of different concentration of Cu on the activities of GPX (A and B), GR (C and D), APX (E and F) and CAT (G and H) in shoot and root respectively after 1st and 5th day of treatment. Data presented are mean ± SE ($n = 3$). *Significant mean difference from control at $P = 0.05$ in multiple comparison by LSD test.

detoxify H_2O_2 using ascorbate for reduction. It is one of the major components of ASH–GSH cycle where it functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms. Thus increase in APX activity with the higher content of GSH and ASH in the Cu treated rice plant of this study might relate with proper functioning of ASH–GSH cycle that detoxifies H_2O_2 thereby preventing from the damage of Cu toxicity. This is in accordance with the findings of Drazkiewicz et al. [25], where ASH–GSH cycle plays an important role in reducing the toxic effect of Cu.

The present study showed that CAT activity did not show significant variation both in shoot and root of the rice plant. CAT is an enzyme which can eliminate H_2O_2 from the plant by degrading it to water and O_2. No definite variation in the CAT activity of the present finding suggests CAT appears not to be required for the elimination of H_2O_2 in the Cu stress rice plant. It might be due to removal of H_2O_2 is carried out by APX and GPX so upgrade in the CAT activity is not required. It has also been reported that CAT activity was not affected by Cu treatment [26]. The response of CAT activity differs broadly depending on the plant species and the metals. Decline in CAT activity was reported in *B. juncea* L. under Cu toxicity [3] whereas enhancement of CAT activity was observed in various plants under several metal toxicity including Cu [27] and Cd [28]. The enhancement in the activities of the antioxidant enzymes is responsible for the scavenging of ROS showing adaptive response of rice plant against Cu stress.

Regarding the present findings, excess Cu induced oxidative stress in rice plant enhancing the ROS production and increased lipid peroxidation. Root suffered more damage than shoot. Stimulation of antioxidant enzymes and antioxidant contents reflects the ability of the rice plant to withstand the Cu induced oxidative stress. Proline accumulation also appears to be an additional defense against Cu oxidative stress.

4. Materials and methods

4.1. Plant cultivation and treatments

Viable rice seed (MSE-9) variety was procured from Regional Agricultural Research Station (AAU), Akbarpur, Karimganj, Silchar. The seeds were surface sterilized with 0.1% mercuric chloride

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($HgCl_2$) solution, rinsed with distilled water and set for germination in clean petri dishes with wetted filter papers at 30 °C in dark. Uniformly germinated seeds were selected and were transferred to the plastic pots containing Hoagland nutrient medium (pH = 6.2). The seedlings were grown in a growth chamber under continuous white light provided with cool, fluorescent white tubes (Philips 20W TLD, India). Copper treatment in the form of $CuSO_4$ of different concentration 10, 50 and 100 μM were given on the 5th day of transferred. Plants were harvested after 1st and 5th day of treatments for various analysis.

4.2. Growth and copper content analysis

Growth of the plant was determined by measuring the length and fresh mass of shoot and root of the plant. The shoot and root were oven dried for 72 h at 80 °C and acid digestion was done as per the method of Humpries [29]. The dry mass (0.1 g) was digested with 5 ml aquaregia in a beaker until dried and made the volume upto 20 ml by adding deionized water and filtered. The total Cu content was measured using atomic absorption spectrometer (*Perkin–Elmer 3110*, Germany).

4.3. Determination of hydrogen peroxide level

The total H_2O_2 content was estimated as per the method of Sagisaka [30]. H_2O_2 was extracted by homogenizing 200 mg of tissue with 5 ml 5% TCA. The homogenate was centrifuged at 12,500 *g* for 10 min. 40 μl of 50% TCA, 20 μl of KSCN and 40 μl of ferrous ammonium sulphate were added to the supernatant to determine H_2O_2 levels. The absorbancy was measured at 480 nm.

4.4. Lipid peroxidation determination

The level of lipid peroxidation is estimated by the method of Heath and Packer [31] in term of MDA content determined by thiobarbituric acid (TBA) reaction. 200 mg of the tissue was homogenized with 5 ml 0.25% TBA. The homogenate was boiled for 30 min at 95 °C and centrifuged at 10,000 *g* for 10 min. The absorbance of the supernatant was recorded at 532 nm and corrected by substracting absorbance at 600 nm.

4.5. Extraction and assay of non-enzymic antioxidant, glutathione and ascorbate

Total glutathione and ascorbate contents were determined as per the method Griffith [32] and Oser [33]. Plant was homogenized with 5 ml phosphoric acid and centrifuged at 17,000 *g* for 15 min at 4 °C. The supernatant was collected and used for assay preparation.

The reaction mixture for ascorbate content contained 2% sodium molybdate, 0.1 N H_2SO_4, 1.5 N Na_2HPO_4 and plant extract. After incubation at 60 °C for 40 min again centrifuged at 3000 *g*. Absorbancy was measured at 660 nm. Glutathione assay mixture contained plant extract, 0.1 M phosphate buffer (pH 6.8), DTNB (5,5-dithiobis-2-nitrobenzoic acid) and the absorbancy was recorded at 412 nm.

4.6. Determination of proline content

Proline concentration was determined following the method of Bates et al. [34]. Plant tissue (0.5 g) was homogenized with 5 ml of sulfosalicylic acid (3%) using mortar and pestle and filtered through Whatman No. 1 filter paper. The volume of filtrate was made up to 10 ml with sulfosalicylic acid and 2.0 ml of filtrate was incubated with 2.0 ml glacial acetic acid and 2.0 ml ninhydrin reagent and boiled in a water bath at 100 °C for 30 min. After cooling the reaction mixture, 6.0 ml of toluene was added, mixed in a vortex mixer and absorbance was read at 570 nm.

4.7. Antioxidant enzymes extraction and assay

For the extraction and estimation of enzymes, plant was homogenized in 0.1 M potassium phosphate buffer, pH 6.8 in a chilled pestle and mortar. The homogenate was centrifuged at 17,000 *g* for 15 min at 4 °C and the supernatant was used for the assay of enzyme activity of SOD, GPX, GR and CAT. For the extraction of APX, 1 mM ascorbate was added to the extraction buffer to restore the activeness of the enzyme.

The assay of SOD was carried out by the method of Gianopolitis and Reis [35]. The assay mixture for SOD (1.15.1.1) contained 79.2 mM Tris–HCl buffer (pH 6.8), containing 0.12 mM EDTA and 10.8 mM tetraethylene diamine, bovine serum albumin (0.0033%), 6 mM nitroblue tetrazolium (NBT), 600 μM riboflavin in 5 mM KOH and 0.2 ml enzyme extract. Reaction was initiated by placing the glass test tubes in between two fluorescent tubes (*Philips 20W*). By switching the light on and off, the reaction was started and terminated, respectively. The increase in absorbance due to formazan formation was read at 560 nm. The activity was expressed as ΔA_{560} (g^{-1} (fr.wt.) min^{-1}).

GPX activity was assayed according to Chance and Maehly [36]. The reaction mixture for GPX (1.11.1.7) contained 2.1 ml of 0.1 M phosphate buffer (pH 6.8), 0.3 ml of 1% guaicol, 0.3 ml of 1% H_2O_2, 0.3 ml of enzyme extract and incubated for 5 min. Absorbancy was taken at 470 nm (extinction co-efficient, $\varepsilon = 26.6$). The activity was expressed as ΔA_{470} (g^{-1} (fr.wt.) min^{-1}).

Glutathione reductase (GR) was assayed by the method of Smith et al. [37]. The reaction mixture of GR (1.6.4.2) contained 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 ml of 3 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) in 0.01 M potassium phosphate buffer (pH 7.5), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract and distilled water to make up a final volume of 2.9 ml. Reaction was initiated by adding 0.1 ml of 2 mM GSSG (oxidized glutathione). The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min spectrophotometrically. The activity is expressed as absorbance change (ΔA_{412}) (g fresh $mass^{-1}$ s^{-1}).

CAT activity was assayed according to Chance and Maehly [36]. The reaction mixture for CAT (1.11.1.6) comprised of 3.0 ml of 0.1 M phosphate buffer (pH 6.8), 1.0 ml of (30 mM) H_2O_2 and 1.0 ml of enzyme extract. The reaction was stopped by adding 10 ml of 2% H_2SO_4 after 1 min incubation at 20 °C and the absorbancy of the reaction mixture was recorded at 240 nm (extinction co-efficient, $\varepsilon = 43.6$). The CAT activity was expressed as unit min^{-1} g (fr.wt).

For APX (1.11.1.11) extraction, according to Nakano and Asada [38], the tissue sample was homogenized with 0.1 M phosphate buffer (pH 6.8) containing 1 mM ascorbate, centrifuged at 17,000 rpm for 15 min at 4 °C. The supernatant was used as enzyme extract for assay mixture. The assay mixture was prepared with 10 mM H_2O_2, 0.5 mM ascorbate, 0.1 M phosphate buffer (pH 6.8), enzyme extract. The absorbancy was taken at 290 nm. With extinction co-efficient 2.8 mM^{-1} cm^{-1}, the APX activity was expressed as ΔA_{290} (g^{-1} (fr.wt.) min^{-1}).

Protein concentrations were estimated according to Bradford [39] using bovine serum albumin as standard.

4.8. Gel electrophoresis

The isoenzyme of SOD was separated by non-denatured electrophoresis on 10% polyacrylamide vertical slab gels under constant current (120 mA) at 4 °C. SOD activity was detected by photochemical staining after electrophoresis according to Ádám et al. [40]. Gel was incubated in a solution containing 50 mM sodium

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phosphate buffer (pH 7.8), 0.1 mM EDTA, 3 mM riboflavin and 0.25 mM nitroblue tetrazolium chloride in the dark for 20 min at 25 °C. After rinsing with distilled water, gels were illuminated under light until the colorless bands were clear against a blue/purple background.

4.9. Statistical analysis

All data obtained were subjected to one-way analyses of variance (ANOVA) and LSD test was used for comparison between mean of treatments. Each bar represents mean ± standard error of three experiments. An asterisk indicates a significant mean difference from control at $P = 0.05$.

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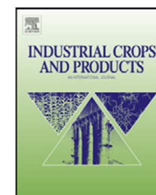

Rapid plant regeneration, analysis of genetic fidelity and essential aromatic oil content of micropropagated plants of Patchouli, *Pogostemon cablin* (Blanco) Benth. – An industrially important aromatic plant

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ABSTRACT

Rapid and prolific shoot regeneration via direct organogenesis was induced from leaf explants of Patchouli, Pogostemon cablin (Blanco) Benth., an aromatic plant of immense industrial value, on Murashige and Skoog (MS) medium with benzylaminopurine (BAP) and α-naphthaleneacetic acid (NAA) within 4 weeks. The adventitious shoot bud induction and plant regeneration were greatly influenced by the origin, age of donor plant, and leaf position on stem. Leaf explants prepared from in vivo plants of different ages showed higher regeneration response as compared to the explants from in vitro plantlets of respective age. The shoot regeneration ability of explants was significantly related to the age of the donor plants as well as the leaf position on the stem. The highest number of shoots (94.6/explants) was obtained from 96.2% of leaf explants derived from the leaves located on the second node of the 3-month-old in vivo plants on Murashige and Skoog's (MS) medium containing 2.5 μM benzylaminopurine (BAP) and 0.5 μM naphthaleneacetic acid (NAA). Incorporation of 1.0 μM gibberellic acid (GA_3) in MS medium significantly improved the shoot elongation (1.8-fold) within 2 weeks in 95% of the cultures. Regenerated shoots rooted spontaneously on growth regulator-free half strength MS medium and were successfully hardened and transferred to nursery with 96–100% survival rate. Genetic fidelity of the in vitro derived plants was assessed using random amplified polymorphic DNA (RAPD). Fourteen arbitrary decamers displayed same banding profile within all the micropropagated plants and in vivo explant donor plant. The molecular analysis complemented and compared well and showed genetic stability in the plants regenerated through direct shoot bud differentiation from leaf explants. The gas chromatogram of the extracted oils from in vitro derived plants and the mother stock plant showed similar essential oil profiles. Rapid and high multiplication frequency, molecular, genetic and essential oil content stability ensure the efficacy of the protocol developed for the production of this industrially important aromatic plant.
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1. Introduction

Essential oil produced by Pogostemon cablin (Blanco) Benth. is one of the most important naturally occurring base materials used in the perfume industry which contains various sesquiterpenes and hydrocarbons such as: Patchouli alcohol (patchoulol), patchoulene, bulnesene, guaiene, caryophyllene, elemene and copaene (Hasegawa et al., 1992). While 92% of the oil is non-odoriferous, the rest is made up of mixture of sesquiterpenes of which norpatchoulenol and (-patchoulene and γ-guaislene are major aroma compounds making it a complex contributing to its characteristic odor. The oil has strong fixative properties and blends very well with oils of sandalwood, geranium, vetiver, clove, etc. giving strength and tenacity for making heavy perfumes of lasting odor. The absence of synthetic substitute for Patchouli oil, which has its characteristic woody fragrance and fixative properties, further enhances its prominent position in the perfume industry, and hence natural oil from cultivation remains the only source of this oil. In addition, Patchouli oil also possesses anti-insecticidal activities (Sharma et al., 1992), anti-fungal and bacteriostatic properties (Kukreja et al., 1990) which are attributed to a number of dehydroacetic acids. Due to its perfumery uses, the demand for Patchouli oil is increasing dramatically worldwide. The *P. cablin* plant is a tropical crop and areas of commercial cultivation are located mainly

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in Indonesia, which accounts for over 80% of world Patchouli oil production. Demand of Patchouli oil in India is large and met at present by import. Considering the growing demand of Patchouli oil than that for most of the other essential oils and availability of large acreage of cultivable land conducive for *P. cablin* plantation in Assam, Kerala, Karnataka and Maharashtra states, India could emerge as a key player in the production of this valuable aromatic oil. It is a vegetatively grown plant being propagated by stem cutting. However, Patchouli mild mosaic virus (PaMMV) and Patchouli mottle virus (PaMoV) along with root knot nematodes (Krishna Prasad and Reddy, 1984) and insect pests (Misra, 1996) are serious pathogens of *P. cablin* which infect the plants and cause decreases in biomass and essential oil yield. Genetic engineering appears to be an effective approach to develop pathogen resistant *P. cablin* plants, and furthermore, offers opportunities to enhance Patchouli oil production with cell culture, callus culture, or plant regeneration. Since *P. cablin* is a fast growing perennial herb, oil production with plant regeneration may be more efficient than with cell culture or callus culture (Jones and Krishnadethan, 1973).

Plant regeneration has been accomplished from protoplast-derived callus (Kageyama et al., 1995), leaf- and node-derived callus (Misra, 1996), and from leaf-derived callus of in vitro derived plants of *P. cablin* (Sugimura et al., 2005). However, no attempts have been made to evaluate the genetic integrity of the in vitro derived plants which is the most important aspect for commercial application of any micropropagation protocol. Considering the fact that the plant regeneration in published protocols are through an intervening callus phase, which can invite genetic instability and somaclonal variation (Gill et al., 2006), and in vitro plants in general are usually susceptible to genetic changes due to culture stress (Dunstan and Thorpe, 1986; Cecchini et al., 1992; Rani and Raina, 1998); the development of a direct plant regeneration system and assessment of clonal fidelity of the in vitro raised plants of *P. cablin* are crucial for successful commercial application of micropropagation protocol. Several techniques such as cytological, isozymes and molecular markers have been employed to detect variation if any or to confirm the genetic stability of micropropagated plants (Gupta and Varshney, 1999). Usefulness of RAPD in detection of variation in micropropagated plants has been amply demonstrated in large array of plants viz., Loblolly pine (Tang, 2001), Chestnut hybrids (Carvalho et al., 2004), *Chlorophytum arundinaceum* (Lattoo et al., 2006), *Hovenia dulcis* (Jeong et al., 2009), *Hypericum perforatum* (Goel et al., 2009) and *Platanus* (Sun et al., 2009)

In this paper, we report an efficient regeneration system via direct multiple shoot bud induction from leaf explants of *P. cablin*, the effect of the origin and age of the donor plant, and physiological age of leaf on adventitious shoot proliferation and plant regeneration. An attempt has been made to assess the clonal fidelity of micropropagated plants by RAPD analysis and to verify essential oil profiles by gas-chromatographic (GC) analysis.

2. Materials and methods

2.1. Plant material and preparation of explants

Young leaves were obtained from two- to 4-month-old shade house-grown plants of Indonesian variety of *P. cablin*. The fully expanded leaves of second and third nodes of the plants were rinsed with a solution of 1% sodium hypochlorite containing 0.1% Tween-20 for 10 min followed by washing under running tap water for 15 min. The leaves were immersed in 70% ethanol for 1 min and surface sterilized with 0.2% (w/v) mercuric chloride for 45 s, and subsequently rinsed thoroughly in sterile distilled water. The leaves were aseptically cut into three segments ($10\ mm^2$) with the leaf edges removed and the leaf segments were used as explants for adventitious shoot bud induction and plant regeneration.

2.2. Multiple shoot induction

The explants were placed with their abaxial surface firmly in contact with MS (Murashige and Skoog, 1962) medium supplemented with 6-benzylaminopurine (BAP) at different concentrations (0.5, 1.0, 2.5, 5.0, and 7.5 μM). The effect of auxin on promotion of multiple shoot induction was evaluated by supplementing α-naphthaleneacetic acid (NAA) at various concentrations (0.5, 1.0, and 2.5 μM) to BAP-containing media.

2.3. Influence of origin, age of donor plant and leaf position on stem, on shoot multiplication

To compare the morphogenic capacity of in vitro explants with their in vivo counterparts, fully expanded leaves were obtained directly from the second and third nodes of the in vivo plants (2-, 3- and 4-month old), and from the micropropagated plantlets, which were previously generated from nodal explants cultured on MS medium containing 1.0 μM BAP. Leaves were aseptically cut into three segments and cultured, as per the procedure described for in vivo leaf segment explants, on MS medium supplemented with 2.5 μM BAP and 0.5 μM NAA.

2.4. Culture conditions

All culture media were supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar-agar (Hi-media, Mumbai, India). The pH of the medium was adjusted to 5.8 prior to autoclaving at 15 psi and 121 °C for 20 min. All the cultures were maintained at 25 ± 2 °C under a 16-h photoperiod with a photosynthetic photon flux density of $35\ \mu mol\ m^{-2}\ s^{-1}$ provided by cool white fluorescent tubes (Philips, India).

After 3 weeks in culture, the efficacy of each medium variant on shoot proliferation and growth was determined by recording (1) regeneration frequency, (2) mean number of shoots per explant, and (3) mean shoot length.

2.5. Shoot elongation, rooting and acclimatization

The explants with shoot clusters, produced after 3 weeks of culture on MS medium containing 2.5 μM BAP and 0.5 μM NAA, were transferred to MS medium supplemented with varied concentrations of gibberellic acid (0.5, 1.0, 2.5, and 5.0 μM) for 2 weeks to allow for the elongation of shoots.

Elongated shoots were separated and cultured for rooting on half strength MS medium without growth regulators. Plantlets with well-developed roots were removed from the culture medium, washed gently under running tap water, and transferred to plastic pots containing soil, vermiculite, and vermicompost (1:1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (28 °C day, 20 °C night, 16-h day length, 70% relative humidity). After a week, the plastic covering was gradually removed and the plantlets were maintained in the greenhouse in plastic pots containing normal garden soil until they were transplanted to the nursery.

The experiments were conducted in a completely randomized design (CRD) and each treatment had three replicates of 20 explants each. To analyze the effect of various treatments, the data were subjected to analysis of variance (ANOVA). Fisher's least significance difference (LSD) test was applied to show statistical differences among the means.

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Fig. 1. Direct adventitious shoot regeneration from leaf explants of Pogostemon cablin (Blanco) Benth. (a) Explant at the time of culture (bar = 10 mm). (b) Adventitious shoot bud formation from the cut-edges of leaf explant within 2 weeks of culture on MS medium + 2.5 μM BAP + 0.5 μM NAA (bar = 10 mm). (c) Microscopic view of leaf edge showing shoot bud induction (bar = 2.5 mm). (d) Proliferation of multiple shoots from the explant edges on MS medium + 2.5 μM BAP + 0.5 μM NAA within 3 weeks of culture (bar = 25 mm). (e) Multiple shoot formation from the explant on MS medium + 2.5 μM BAP + 0.5 μM NAA within 4 weeks of culture. (f) Prolific elongation of shoots from primary cultures within 2 weeks of culture on MS + 1.0 μM GA_3. (g) A rooted shoot on half strength MS medium. (h) Plants after acclimatization in the greenhouse. (i) Plants established in nursery.

2.6. RAPD fingerprinting: preparation of genomic DNA and PCR amplification

Genomic DNA was isolated from leaves of randomly selected hardened in vitro plants after 3 months of their transfer to field conditions. DNA was also extracted from in vivo mother plant. DNA was isolated following the modified CTAB procedure (Rogers and Bendich, 1985) and the yield and purity were checked by electrophoresis in a 0.8% (w/v) agarose gel and also from the value obtained by UV absorbance ratio at 260 nm/280 nm.

In a pre-screen with 26 primers based on amplification of mother plant, 14 arbitrary decamer primers (Operon Technologies, USA) were selected for polymerase chain reaction (PCR). These primers produced distinct amplification profiles that were easily scorable. DNA amplification was performed in Thermal Cycler (Applied Biosystems, Model 2720) and the PCR conditions were as follows: 5 min at 94 °C; 35 cycles of: 1 min at 94 °C, 1 min at 30 °C, 1 min at 72 °C; and a final extension step 7 min at 72 °C. The 25-μl reaction mixture contained 50 ng of template DNA, 200 μM dNTP (Bangalore Genei, Bangalore, India), 50 pmol decamer random primers, 1× Taq DNA polymerase buffer containing Tris with 15 mM $MgCl_2$ (Bangalore Genei, Bangalore, India) and 1 unit of Taq DNA polymerase (Bangalore Genei, Bangalore, India). The amplified products were resolved by electrophoresis in 1.2% (w/v) agarose gels run in 1× TBE and visualized under UV light and documented using the Gel Documentation equipment (BioRad, Hercules, CA). All the PCR reactions were repeated at least twice to check the reproducibility.

2.7. Evaluation of essential oils extracted from regenerated plants

The Patchouli essential oils were extracted by steam distillation of shade dried leaves (100 g) of the in vivo mother stock (control) plant and randomly selected in vitro derived 3-month-old plants by using Clevenger's apparatus. The oil samples were collected and dried over anhydrous Na_2SO_4 and stored at 4 °C until analyzed. The

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Table 1
Effect of different concentrations of benzylaminopurine (BAP) alone or in combination with different concentrations of naphthaleneacetic acid (NAA) on adventitious shoot proliferation and mean number of shoots from leaf explants of Pogostemon cablin cultured on MS medium after 3 weeks.

Plant growth regulators (μM)		Regeneration frequency (%)	Mean number of shoots/explant	Mean shoot length (cm)
BAP	NAA			
0.5	0.0	34.1^{ef}	11.6 ± 0.28^{e}	2.8 ± 0.11^{a}
	0.5	45.4^{de}	20.3 ± 0.31^{de}	2.1 ± 0.08^{ab}
	1.0	39.2^{ef}	14.8 ± 0.19^{e}	1.8 ± 0.05^{b}
	2.5	37.5^{ef}	13.5 ± 0.23^{e}	1.5 ± 0.07^{bc}
1.0	0.0	51.2^{de}	25.1 ± 0.16^{d}	1.7 ± 0.04^{b}
	0.5	61.6^{cd}	38.0 ± 0.22^{cd}	1.4 ± 0.09^{bc}
	1.0	55.3^{cd}	31.4 ± 0.41^{d}	1.2 ± 0.03^{bc}
	2.5	53.7^{cd}	28.3 ± 0.17^{d}	0.9 ± 0.05^{c}
2.5	0.0	67.3^{bc}	62.2 ± 0.24^{bc}	1.3 ± 0.02^{bc}
	0.5	82.2^{a}	81.3 ± 0.30^{a}	1.0 ± 0.04^{c}
	1.0	73.6^{b}	70.7 ± 0.25^{b}	0.9 ± 0.06^{c}
	2.5	71.1^{bc}	64.4 ± 0.18^{bc}	0.8 ± 0.02^{c}
5.0	0.0	19.3^{fg}	43.1 ± 0.23^{cd}	0.8 ± 0.04^{c}
	0.5	26.7^{f}	54.3 ± 0.20^{bc}	0.7 ± 0.03^{c}
	1.0	24.5^{fg}	51.2 ± 0.17^{c}	0.7 ± 0.02^{c}
	2.5	20.1^{fg}	44.7 ± 0.21^{cd}	0.6 ± 0.03^{c}
7.5	0.0	11.4^{g}	34.3 ± 0.32^{cd}	0.6 ± 0.04^{c}
	0.5	18.3^{fg}	42.6 ± 0.19^{cd}	0.6 ± 0.02^{c}
	1.0	13.6^{g}	35.2 ± 0.27^{cd}	0.5 ± 0.06^{cd}
	2.5	12.5^{g}	34.9 ± 0.22^{cd}	0.5 ± 0.04^{cd}

The values are the means (±SE) of three replicates with 20 explants each. Means within a column followed by different letters differ significantly at $P \leq 0.05$ as compared through ANOVA and LSD test.

quantitation of the essential oil was done by measuring the volume of oil recovered, in a collection tube. The oil concentration in plants was expressed as percentage on a volume basis (ml of oil obtained from 100 g of fresh herbage). The oil samples were analyzed for their composition using gas chromatography. Gas chromatography was performed on a Thermo-Chemito Model 1000 instrument equipped with Flame Ionization Detector (FID) and programme temperature vaporizer (PTV), using a fused silica capillary column BP-5 (30 m × 0.32 mm id, 0.25 μm film thickness). The GC column oven was programmed as 80–225 °C (5 min) at 3 °C/min. The PTV injector port (split mode) was also programmed from 120 °C to 240 °C at fast programmed rate with split ratio of 1:20; whereas the detector temperature was maintained at 240 °C. The carrier gas (helium) flow was maintained at flow rate of 2 ml min^{-1} with make-up gas at 25 ml min^{-1}. An IRIS 32 data processor, coupled with the instrument was used for area percentage calculation of the total eluted peaks. Components were identified with standard compounds by comparing the retention times and with co-injection techniques, under similar operating parameters. The presence of the major compounds was further confirmed by GCMS using NIST (National Institute of Standards and Technology, USA) and Wiley database libraries available with the GCMS software system.

3. Results and discussion

3.1. Multiple shoot induction from leaf segments

Young leaf segments prepared from leaves (Fig. 1a) of 3-month-old shade house-grown plants on culture on MS medium supplemented with BAP or/and NAA, got curled and swelled at their edges after a week of incubation showing the sign of dedifferentiation. Adventitious shoot bud formation was observed from the cut-edges of leaf tissues within 2 weeks of initiation of culture (Fig. 1b and c). Multiple shoots were clearly visible arising from the explant edges within a week of bud induction with a little or no callus in the explants (Fig. 1d). Sarwar and Skirvin (1997) proposed that the cut-edges of leaves provided a way for nutrients and growth regulators to be absorbed efficiently from the medium. With increase in the concentration of BAP from 0.5 to 2.5 μM in the media, there was gradual increase in the percentage of multiple shoots forming explants (34.1–67.3%) and the average number of shoots per explant (Table 1). The dosage of cytokinin in the culture medium is known to be critical for shoot organogenesis (Sun et al., 2009). The promotory effect of BAP in inducing multiple shoots has been previously reported in *Garcinia mangostana* (Goh et al., 1990; Huang et al., 2000; Sun et al., 2009). However, the elevated concentration of BAP reduced the percent of explants forming multiple shoots and average number of shoots per explant and furthermore, induced callusing at the cut ends to varying degree. The effect of auxin (NAA) in combination with BAP at different concentrations was assessed. The addition of NAA to BAP in medium increased the frequency of multiple shoot formation and average number of shoots per explant (Table 1). Lower levels of BAP with NAA were less effective in generating shooting response from leaf explants. It was observed that there was a considerable increase in explants forming multiple shoots and shoot number on lower NAA and higher BAP levels, while higher levels of auxin in the BAP-containing media lead to decline in shoot number. The best morphogenic response was observed on media supplemented with 0.5 μM NAA and 2.5 μM BAP, enhancing the frequency of multiple shoot forming explants from 67.3% to 82.2%, and the average number of shoots from 62.2 to 81.3 (Table 1 and Fig. 1e). The fact that NAA treatment could possibly eliminate the secretion of phenolic substances by competing for the active sites of the auxin oxidase enzyme involved in oxidization of phenols (Perez-Tornero et al., 2000), might have played a role in promoting BAP in shoot induction from leaf disc explants of Patchouli known to secrete essential oils and phenols (Sugimura et al., 2005). The presence of NAA along with BAP in the medium has been reported to further improve the shoot induction from leaf disc explants in *Curculigo orchioides* (Thomas, 2007), *Moricandia arvensis* (Rashid et al., 1996), *Spilanthus acmella* (Saritha and Naidu, 2008), *Dianthus chinensis* (Kantia and Kothari, 2002), *Dianthus caryophyllus* (Van Altvorst et al., 1992, 1994, 1995), and in essential oil yielding plant such as *Mentha arvensis* (Phatak and Heble, 2002), *Ocimum basilicum* (Dode et al., 2003), in Zonal (*Pelargonium* × *hortorum*) and two scented

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Table 2
Influence of the origin and age of the donor plant, and leaf position on node on adventitious shoot proliferation and mean number of shoots from leaf explants of Pogostemon cablin cultured on MS + 2.5 μM BAP + 0.5 μM NAA after 3 weeks.

Origin of donor plant	Age of donor plant (months)	Position of leaf on the node of plant (from shoot apex)			
		Second node		Third node	
		Regeneration frequency (%)	Mean number of shoots/explant	Regeneration frequency (%)	Mean number of shoots/explant
Ex vitro	2	82.3^{b}	81.0 ± 0.23^{b}	63.4^{cd}	71.5 ± 0.29^{bc}
	3	96.2^{a}	94.6 ± 0.34^{a}	72.3^{c}	80.4 ± 0.31^{b}
	4	53.1^{de}	65.2 ± 0.28^{c}	42.5^{e}	51.7 ± 0.23^{d}
In vitro	2	60.6^{d}	66.1 ± 0.31^{c}	54.2^{de}	56.6 ± 0.34^{d}
	3	71.2^{c}	75.6 ± 0.36^{bc}	62.4^{cd}	67.3 ± 0.41^{c}
	4	50.4^{de}	52.3 ± 0.27^{d}	38.1^{e}	41.4 ± 0.35^{e}

The values are the means (±SE) of three replicates with 20 explants each. Means within alternate column followed by different letters differ significantly at $P \leq 0.05$ as compared through ANOVA and LSD test.

(*P. capitatum* and *P. graveolens*) Geraniums (Hassanein and Dorion, 2005). However, a further increase in NAA concentration to media containing 2.5 μM BAP could not improve its synergistic effect on regeneration (Table 1). In the case of Patchouli leaf explants, the influence of nutrient media could not be assessed as explants failed to respond morphogenically to growth regulator-free media.

3.2. Effect of origin and age of donor plant material, and physiological age of the leaves on regeneration

The regeneration response was significantly different among explants from leaves of plants of in vivo and in vitro origin, as well as for plants of different ages (Table 2). Furthermore, the position of the leaves on the stem of the mother stock plant exerted a significant difference in regeneration response from leaf segment explants. Compared to the explants of in vitro origin, explants of in vivo origin of respective age were found significantly more productive for multiple shoot formation (Table 2). The competence for shoot production on in vivo leaf explants has been reported to be significantly higher than on in vitro leaf explants in *Passiflora edulis* (Becerra et al., 2004) and cottonwood hybrid, genus *Populus* (Han et al., 2000). In case of explants of in vitro origin, micropropagation might have caused a state of low morphogenic capacity in leaf tissues, which may be revealing the effect of the in vitro environment (high relative humidity, low light intensity, and medium composition).

The age of the plant material played a profound role on determining the competence of the leaf explants for efficient adventitious shoot proliferation in *P. cablin*. Among the different age plants tested, explants from 3-month-old in vivo plants induced significantly higher multiple shoots (Table 2). Explants from different age plants may have different levels of endogenous hormones and, therefore, the age of explants would have a critical impact on the regeneration success, similar results have been reported in other plants, including *Platanus occidentalis* (Sun et al., 2009), *Morus alba* (Thomas, 2003), *Cercis canadensis* (Distabanjong and Geneve, 1997), *Malus* (Famiani et al., 1994), *Cydonia oblonga* (Baker and Bhatia, 1993), *Aegle marmelos* (Islam et al., 1993), *Lachenalia* (Niederwieser and Van Staden, 1990) and *Prunus* (Mante et al., 1989).

A significant difference in the frequency of multiple shoot induction and mean shoot number was recorded between the explants of leaves located at different nodal positions on the plant. The leaf explants of second node, which originally located closer to the stem apex, showed a higher percentage of shoot regeneration than the explants of third node (older explants). A similar gradient response was also found in the number of shoots per regenerating explant. Leaf explants of second node produced more shoots than explants of third node (Table 2). The leaf explants of second node showed best multiple shoot induction response, irrespective of the age and nature of origin of the plant material (Table 2). This could be a kind of age-related response, because the position was related to the physiological age of the explants, and the closer to the apex the younger the explant. Intriguingly, no regeneration response was observed from leaf explants of first node of in vivo- as well as in vitro-originated plants (data not shown). The highest regeneration response was obtained in leaf explants located in the second node of the 3-month old in vivo plants (Table 2).

Leaves have been most widely studied for adventitious shoot formation in diverse plants. The observations regarding the importance of position of leaves on the plant agree with what has been observed by Sankhla et al. (1993) in *Dianthus* who reported that the youngest leaves just below the apical meristem, when used as explants, gave the highest regeneration response, and the regeneration response declined towards the third pair of leaves. Similar observations have also been reported in *Prunus serotina* (Liu and Pijut, 2008), *P. armeniaca* (Perez-Tornero et al., 2000) and *Decalepis arayalpathra* (Sudha et al., 2005). Famiani et al. (1994) proposed that the higher regeneration rates of apical leaves in apple might be because the youngest leaves, still developing, have less differentiated and more metabolically active cells, with a more suitable hormonal and nutritional situation that could improve organogenesis. In spite of this, Antonelli and Druart (1990) obtained the best results in apple using older leaves. Contrasting regenerative capac-

Table 3
Effect of gibberellic acid (GA_3) on elongation of shoots from leaf explants of Pogostemon cablin on MS medium after 2 weeks of culture.

GA_3 (μM)	Shoot elongation response (%)	Mean shoot length (cm)	Fold increase in shoot length
–	0	1.0 ± 0.12^{c}	0
0.5	77^{b}	1.9 ± 0.13^{b}	0.9 ± 0.02^{b}
1.0	95^{a}	2.8 ± 0.16^{a}	1.8 ± 0.05^{a}
2.5	56^{c}	1.7 ± 0.11^{b}	0.7 ± 0.03^{b}
5.0	51^{c}	1.3 ± 0.15^{bc}	0.3 ± 0.01^{c}

The values are the means (±SE) of 3 replicates with 20 explants each. Means within a column followed by different letters differ significantly at $P \leq 0.05$ as compared through ANOVA and LSD test.

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Table 4
Summary of the statistics of RAPD analysis of plants regenerated from leaf explants of *P. cablin*.

Primer name	Primer sequence	No. of amplicons	Size range of amplified products (kb)
OPU-08	5′-GGCGAAGGTT-3′	4	0.3–1.5
OPU-17	5′-ACCTGGGGAG-3′	3	0.4–0.6
OPQ-07	5′-CCCCGATGGT-3′	3	0.5–2.3
OPQ-09	5′-GGCTAACCGA-3′	2	0.8–1.5
OPQ-13	5′-GGAGTGGACA-3′	4	0.8–2.06
OPQ-16	5′-AGTGCAGCCA-3′	2	0.5–0.6
OPG-02	5′-GGCACTGAGG-3′	4	0.3–2.06
OPN-11	5′-TCGCCGCAAA-3′	3	0.8–1.05
OPN-16	5′-AAGCGACCTG-3′	7	0.4–1.5
OPN-18	5′-GGTGAGGTCA-3′	1	0.8
OPS-01	5′-CTACTGCGCT-3′	3	0.7
OPS-02	5′-CCTCTGACTG-3′	2	0.5–0.6
OPS-04	5′-CACCCCCTTG-3′	2	0.8–1.5
OPT-08	5′-AACGGCGACA-3′	4	0.6–2.3
Total number	14 primers	44	0.3–2.3

ity of the different segments of leaf have been reported in apple (Welander, 1988; Yepes and Aldwinckle, 1994), *Piper colubrinum* (Kelkar and Krishnamurthy, 1998) and *Hagenia abyssinica* (Feyissa et al., 2005) indicating the higher regeneration capacity from the middle part to the base as compared to apex, and further explaining the reason that leaves reach maturity first at the apex and subsequently in a basipetal progression. However, such differential regeneration capacity was not observed in our case. It could be attributed to the effect of polarity on regeneration potential which is a complex process that includes both transport of hormones and tissue maturity (Feyissa et al., 2005).

3.3. *Shoot elongation*

In the present study, BAP and NAA together induced higher multiple shoot bud induction and proliferation, however, the multiple shoots obtained on all combinations of BAP and NAA took prolonged duration for elongation in culture, showing elongation response after 12 weeks of initial culture of explants. The elongation could not be accelerated upon transferring the cultures to phytohormone-free MS basal medium (data not shown). Hence, it was necessary to develop a suitable medium for faster elongation of shoot clumps. Incorporation of 1.0 μM GA_3 to MS media enhanced the shoot elongation by 1.8-fold in 95% of shoot cluster cultures within 2 weeks (Table 3 and Fig. 1f), whereas no shoot elongation was observed in medium devoid of GA_3, in 2 weeks duration (Table 3). The promotive effect of GA_3 on elongation of stunted shoots, generated on BAP-containing medium, has been reported in several other plant species (Purkayastha et al., 2008; Sugla et al., 2007; Purohit and Singhvi, 1998; Jordan and Oyanedel, 1992; Demeke and Hughes, 1990). GA_3 is considered to stimulate shoot elongation by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger, 1998).

3.4. *Rooting of shoots and acclimatization*

Regenerated shoots with four to six leaves were rooted on the growth regulator-free half strength MS medium. The percentage of rooting was 100% and 8–10 roots per shoot induced after 1 week on root induction medium (Fig. 1g). Plantlets with four to six fully expanded leaves and well-developed roots were successfully acclimatized in the greenhouse, in pots containing soil, vermiculite and vermicompost (1:1:1) within 2 weeks (Fig. 1h) and eventually established in a nursery (Fig. 1i) with a survival frequency of 96–100%. The established plants were apparently uniform and did not show any detectable variation.

3.5. *RAPD analysis*

Utility of RAPD as a means of evaluation of clonal fidelity and genetic stability of in vitro regenerated plants has been very well documented by many workers (Chawdhury and Vasil, 1993; Rout et al., 1998; Das and Pal, 2005; Agnihotri et al., 2009; Lattoo et al., 2006; Chaudhuri et al., 2007, 2008; Sun et al., 2009). Genetic fidelity of micropropagated plants has immense practical utility and commercial implications. Furthermore, RAPD is referred as an appropriate tool for certification of genetic fidelity of in vitro propagated plants (Gupta and Rao, 2002). Keeping this perspective in mind, we assessed the genetic integrity of in vitro regenerated plants through multiple bud induction in *P. cablin*.

The RAPD band pattern of randomly selected in vitro derived plants was compared with the donor mother plant. Fourteen

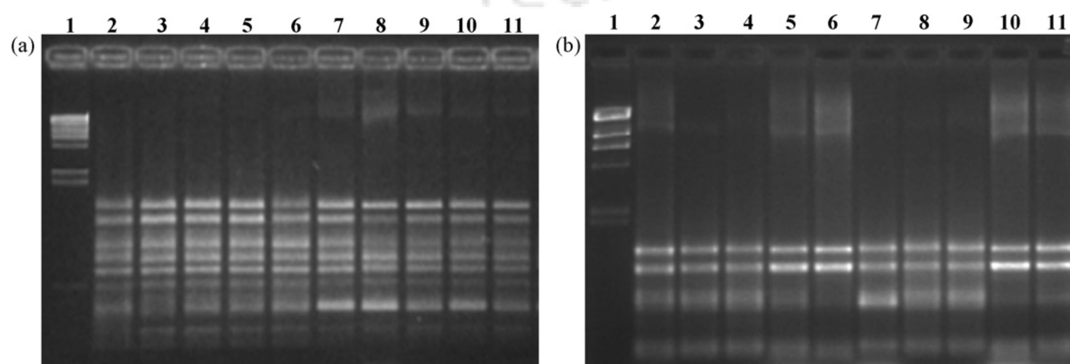

Fig. 2. DNA fingerprinting patterns generated with primers OPN-16 (a) and OPU-08 (b) among regenerants when compared with the donor plant: donor plant (lane 2), micropropagated plants (lanes 3–11), and molecular weight marker lambda DNA HindIII (lane 1).

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Table 5
Essential oil content and components in *P. cablin* in vitro derived plants[a] and in vivo donor plant.

Oil component	Retention time (min)	Content[b] (% of essential oil)	
		In vitro	In vivo
δ-Patchoulene	09.36	0.11 ± 0.02	03.68 ± 0.15
β-Elemene	12.35	0.87 ± 0.04	01.45 ± 0.08
Caryophyllene	13.87	2.03 ± 0.13	02.12 ± 0.11
α-Guaiene	14.72	6.75 ± 0.48	09.79 ± 0.98
Seychellene	14.81	5.11 ± 0.61	06.18 ± 0.22
β-Patchoulene	15.27	3.50 ± 0.13	04.41 ± 0.14
δ-Guaiene	17.27	1.65 ± 0.04	01.67 ± 0.03
α-Bulnesene	17.58	11.50 ± 1.12	10.92 ± 1.62
Caryophyllene oxide	20.46	0.58 ± 0.03	0.31 ± 0.02
Pogostol	22.32	0.31 ± 0.02	0.74 ± 0.06
Patchouli alcohol	23.46	56.30 ± 1.32	44.35 ± 1.84

[a] Plants regenerated by adventitious shoot proliferation from leaf explants cultured on MS + 2.5 μM BAP + 0.5 μM NAA.
[b] The values are means (±SE) of three replicates.

primers (Table 4) that produced distinct amplification profiles, displayed same banding pattern in all the in vitro derived plants as the DNA sample from donor mother plant. The representative profiles of the nine in vitro derived plants and the control donor mother plant with two primers (OPN-16 and OPU-08) are shown in Fig. 2a and b. A total of 44 amplification products were detected with an average of 3.14 fragments per primer. Each primer produced a unique set of amplification products, ranging in size from 0.3 kb (OPU-08, OPG-02 primers) to 2.3 kb (OPQ-07, OPT-08 primers). The monomorphic banding pattern in micropropagated plants and in vivo mother plant in *P. cablin* possibly indicated that organized direct multiple shoot bud formation from leaf explants without intervening callus phase is least vulnerable to genetic changes. This establishes the suitability of protocol developed for the micropropagation of *P. cablin*.

3.6. Essential oil analysis

The essential oil content in the in vitro derived plants was determined to be 4% as compared to 3.2% detected in in vivo plants. The major essential oil constituents detected in in vitro derived plants and in vivo plants were similar and included Patchouli alcohol, Pogostol, caryophyllene oxide, α-bulnesene, δ-guaiene, δ-patchoulene, seychellene, α-guaiene, caryophyllene, β-elemene and β-patchoulene. However, the content of the constituents were found differing between in vitro derived plants and in vivo plants (Table 5). The percent contents of Patchouli alcohol (56.30) in the in vitro derived plants were found significantly higher than that of in vivo plants (44.35). Several studies have established that the content of essential oils detected in in vitro plants was higher than their in vivo counterparts. Arafeh (1999) reported that the essential oil content of in vitro-grown *Origanum vulgare* was sixfold more than that in in vivo plants. Similarly, the essential oil content in the in vitro derived plants (0.7%) was found significantly higher than in vivo plants (0.34%) of *Salvia fruticosa* (Arikat et al., 2004). On the contrary, Hirata et al. (1990) obtained almost equal amounts of essential oils from in vitro plantlets and in vivo mother stock plants of *Mentha spicata*. The difference in the essential oil content between in vitro derived plants and in vivo stock plants could be attributed to the fate of glandular trichomes to which the synthesis of monoterpene is restricted (Arikat et al., 2004). Timing of gland ontogeny was shown to directly influence the concentration of monoterpenes (Gershinzon et al., 1989). Under in vitro conditions, harvested shoots are young and thus chemical compounds that are secreted by the trichomes accumulate in the shoot and their amount is concentrated. Furthermore, the increased yield of essential oils in in vitro derived plants may be related to the marked

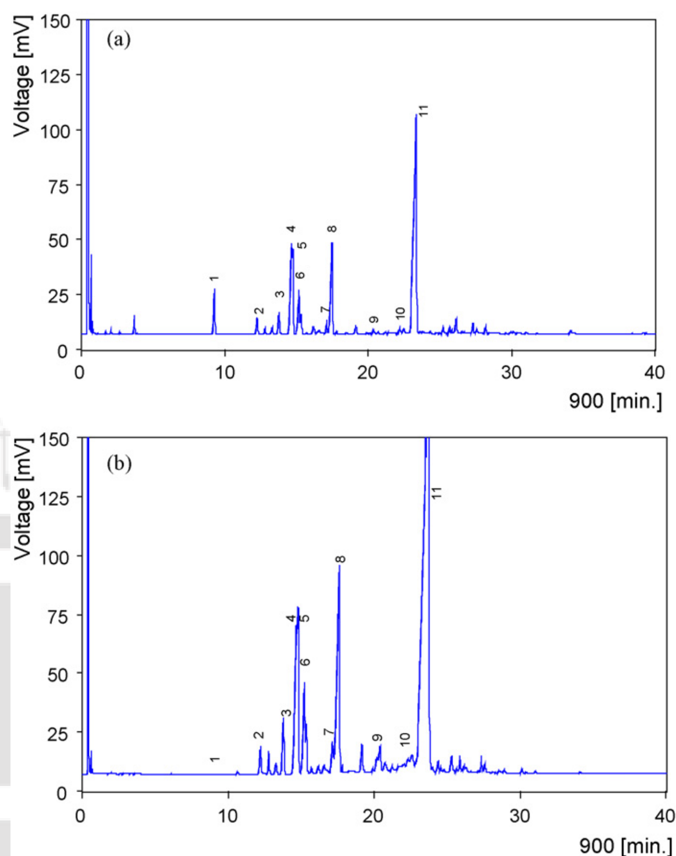

Fig. 3. Gas-chromatographic profiles of essential oils extracted from in vivo donor plant (a) and in vitro derived plant (b) of Pogostemon cablin (Blanco) Benth. Peaks (1) δ-patchoulene, (2) β-elemene, (3) caryophyllene, (4) α-guaiene, (5) seychellene, (6) β-patchoulene, (7) δ-guaiene, (8) α-bulnesene, (9) caryophyllene oxide, (10) Pogostol, (11) Patchouli alcohol (along *X*-axis: retention time in min, along *Y*-axis: detector response in mV).

influence of cytokinin in the culture medium. Benzyladenine (BA) in the medium was shown to have a positive effect on the capacity of *Lavandula dentata* plantlets to produce and/or accumulate essential oils; the amount was 150% more than that produced in the absence of BA (Sudria et al., 1999). The authors attributed this result to the increased percentage of glandular hairs at the secretory stage as a result of addition of BA to the medium and not to the increased biomass of the culture. The GC profiles (Fig. 3a and b) of the essential oil from in vitro derived plants and in vivo plants was found similar with major peaks at retention times of 23.46 and 23.87 min respectively, which may be of the component Patchouli alcohol that formed the primary component of the Patchouli essential oil.

4. Conclusion

In conclusion, an efficient procedure was developed for direct shoot regeneration from the leaf explants of *P. cablin*, an industrially important aromatic plant. The factors that significantly influenced the efficiency of prolific adventitious shoot regeneration were the origin and age of donor plant material, and position of leaf on stem. High frequency of multiple shoot induction and significantly higher mean number of shoots were obtained from leaf segment explants prepared from the leaves of second nodes of 3-month-old in vivo plants. Furthermore, genetic fidelity of micropropagated plants was assessed employing RAPD. The molecular analysis showed the genetic integrity and stability in the plants developed through direct shoot bud differentiation from leaf explants. The essential oil content analysis of micropropagated plants was found sim-

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ilar to that of in vivo mother stock plants. High multiplication frequency, genetic stability and essential oil content uniformity ensures the efficacy of the protocol developed for homogenous production of this industrially important aromatic plant. In the future, using biotechnological approaches should enable the introduction of candidate genes for useful traits, such as virus and nematode resistance. Although biosynthetic pathways of sesquiterpenes in Patchouli plants are not fully understood yet, metabolic engineering approaches may make it possible to increase the content of Patchouli alcohol (a key component of Patchouli oil) and to alter the constituents of Patchouli oil, thereby providing the new essential oils with unique odor. In our laboratory, experiments are in progress to evaluate critical factors affecting efficient *Agrobacterium*-mediated gene transfer into *P. cablin*.

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Research article

Excess copper induced oxidative stress and response of antioxidants in rice

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ABSTRACT

To investigate the effects of copper (Cu), rice plant (*Oryza sativa*. L. var. MSE-9) was treated with different Cu concentrations (0, 10, 50 and 100 μM) for 5 days in hydroponic condition. Gradual decrease in shoot and root growth was observed with the increase of Cu concentration and duration of treatment where maximum inhibition was recorded in root growth. Cu was readily absorbed by the plant though the maximum accumulation was found in root than shoot. Hydrogen peroxide (H_2O_2) production and lipid peroxidation were found increased with the elevated Cu concentration indicating excess Cu induced oxidative stress. Antioxidant enzymes superoxide dismutase (SOD), guaiacol peroxidase (GPX) and ascorbate peroxidase (APX) and glutathione reductase (GR) were effectively generated at the elevated concentrations of Cu though catalase (CAT) did not show significant variation with respect to control. Ascorbate (ASH), glutathione (GSH) and proline contents were also increased in all the Cu treated plants compared with the control. SOD isoenzyme was greatly affected by higher concentration of Cu and it was consistent with the changes of the activity assayed in solution. The present study confirmed that excess Cu inhibits growth, induced oxidative stress by inducing ROS formation while the stimulated anti-oxidative system appears adaptive response of rice plant against Cu induced oxidative stress. Moreover proline accumulation in Cu stress plant seems to provide additional defense against the oxidative stress.
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1. Introduction

Heavy metal toxicity is one of the major environmental problems to the present world because of its increasing level caused by both natural sources (e.g., wind-blown dust, decaying vegetation, forest fires and sea spray) and human activities (e.g., mining, metal production; wood production and phosphate fertilizer). Even in trace concentration, they caused serious problem to all the organisms. Among the heavy metals, copper (Cu) is an essential micronutrient for plant growth and various biochemical processes. It is required in much important biological function since they are constituents of many enzymes and proteins. It is a co-factor of enzymes like plastocyanin, cytochrome c, and Cu/Zn^- superoxide dismutase (Cu/Zn–SO). However, excessive Cu adversely affects plant growth and metabolism. Even slightly higher concentration of Cu from the optimal level induces toxicity to the plant [1]. Being a redox active metal, Cu catalyzes the production of reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH•), via Haber–Weiss and Fenton reactions [2]. ROS are damaging to essential cellular components such as DNA, proteins and lipids therefore induction of ROS production led to oxidative stress affecting plant growth and alteration of antioxidant system [3].

Plants have evolved certain mechanisms to tolerate heavy metal stress such as metal exclusion, metal accumulation and binding of heavy metal by strong ligands like cysteine-rich proteins, metallothioneins (MTs) and thiol-rich peptides, phytochelatins (PCs) [4,5]. Moreover, plants have stimulated antioxidant system to combat the oxidative injury induced by the heavy metals [6]. These include several ROS-removing enzymes such as superoxide dismutase (SOD), catalases (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), and low molecular mass antioxidants scavengers such as ascorbate (ASC) and glutathione (GSH). It has been suggested that Cu induced antioxidative reactions in the roots of *Brassica juncea* and in Arabidopsis [4]. These induced cellular antioxidants scavenged ROS thereby preventing the damage caused by the overproduction of ROS. Generation of proline is also one of the vital responses of plant under Cu toxicity which is possibly associated with the protection

Abbreviations: APX, ascorbate peroxidase; ASH, ascorbate; CAT, catalase; GPX, guaiacol peroxidase; GSH, glutathione; GR, glutathione reductase; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; TBA, thiobarbituric acid; TCA, trichloroacetic acid; H_2O_2, hydrogen peroxide.

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of plant cells against oxidative damage and with signal transduction [7]. Proline might protect plants from metal toxicity by chelating heavy metals in the cytoplasm or as a hydroxyl radical scavenger [8]. It can be considered as a non-enzymic antioxidant which is involved in counteracting the ROS damage. However different plants, plant parts and metals evolved different strategies to resist the toxicity.

Rice is an important staple food of the world population containing high nutritional quality among cereal crops. Half of the world population depends on rice for survival but due to certain heavy metal toxicity including Cu caused reduction of the crop yield. The present investigation was undertaken to test the effects of Cu on growth, induction of oxidative stress and antioxidative responses in rice (*Oryza sativa* L.) variety MSE-9.

2. Results

2.1. Changes of growth and copper uptake

The effect of different Cu concentrations on the growth of rice plant was given in Table 1. A gradual decrease in shoot and root elongation was observed with the increase in Cu concentration and length of treatment. Maximum inhibition was occurred at 100 μM Cu after 5th day in shoot (18.84%) and root (27.59%) with respect to control. At the same concentration, fresh mass of shoot is decreased by 35.31% while in root 44.44% is found to be decreased (Table 1) suggesting higher impact of Cu toxicity in root than shoot. From the result, it also seen that biomass is more susceptible to Cu toxicity than length. Cu content in root and shoot (Table 1) increased gradually with increasing Cu concentrations in the treatment though root showed greater Cu content than shoot.

2.2. Hydrogen peroxide level

The level of H_2O_2 increased progressively both in shoot and root with the increase of Cu concentrations and duration of treatment (Fig. 1A, B). Maximum increase was observed at the highest concentration (100 μM) after 5th day where shoot showed 32.82% increment in comparison with control. Much higher levels were observed in root with 44.07% and 50.11% increased over the control exposed to 100 μM Cu after 1st day and 5th day respectively. The H_2O_2 production was quite proportional with concentration and duration of Cu treatment.

2.3. Lipid peroxidation

Lipid peroxidation measured in terms of MDA increased in all the stressed plants comparing with control. MDA content in shoot and root gradually increased with the increase of Cu concentrations (Fig. 1C, D) and its content was more in root than that of shoot in all the experimental condition. Significant increase of MDA content was found at 100 μM Cu in root after 5th day of treatment (43.82% over control). The increase in MDA content under Cu treatment can be interrelated with an increase in concentrations and duration of treatment.

2.4. Ascorbate and glutathione contents

Treatment of the rice plant with different concentrations of Cu increases ASH content in both shoot and root (Table 1). The ASH contents increased by 24.13% in shoot while 29.31% in root at 100 μM after 5th day of treatment over control. GSH content was also enhanced in shoot and root of 10, 50 and 100 μM of Cu with respect to control (Table 1). Maximum increase was observed at 100 μM Cu after 5th day of treatment by 44.20% in shoot while 54.25% in root.

2.5. Proline content

As shown in Table 1, proline content increased with the increasing Cu concentrations in the treatment. The proline content increased by 26.89%, 45.58% and 67.60% in shoot while 19.12%, 54.81% and 110.43% in root at 10, 50 and 100 μM Cu respectively with respect to control after 1st day of treatment. Almost similar

Table 1
Effect of different concentrations of Cu on growth, fresh mass, contents of Cu, ASH, GSH and proline in shoots and roots of rice plant after 1st day and 5th day of treatment. The data presented are mean of three separate experiments ± SE. * indicates significant mean difference from control at $P = 0.05$ according to LSD test.

Parameter		0 μM	10 μM	50 μM	100 μM
Shoot					
Growth (cm)	1st day	14.65 ± 0.579	13.58 ± 0.630	13.10 ± 0.826	12.93 ± 0.866
	5th day	16.88 ± 0.781	16.12 ± 0.758	14.60 ± 0.720	13.70 ± 0.917
Fresh mass (g)	1st day	0.266 ± 0.033	0.235 ± 0.038	0.194 ± 0.038	0.184 ± 0.038
	5th day	0.354 ± 0.029	0.324 ± 0.030	0.317 ± 0.029	0.229 ± 0.032*
Cu [$\mu g\ g^{-1}$ (f.m.)]	1st day	0.049 ± 0.007	0.098 ± 0.005	0.125 ± 0.008	0.298 ± 0.008*
	5th day	0.044 ± 0.012	1.812 ± 0.317	3.660 ± 0.234*	4.874 ± 0.265*
Ascorbate [$\mu mol\ g^{-1}$ (f.m.)]	1st day	569.40 ± 61.97	588.87 ± 40.19	613.65 ± 59.70	673.63 ± 61.26
	5th day	573.97 ± 58.54	596.37 ± 52.14	639.98 ± 57.26	712.45 ± 64.72
Glutathione [$\mu mol\ g^{-1}$ (f.m.)]	1st day	2537.45 ± 217.25	2733.74 ± 228.16	2591.07 ± 222.69	3725.01 ± 224.11*
	5th day	2855.58 ± 192.97	2907.16 ± 193.29	3445.10 ± 204.12	4117.92 ± 190.69*
Proline [$\mu mol\ g^{-1}$ (f.m.)]	1st day	2.034 ± 0.238	2.581 ± 0.301	2.961 ± 0.320	3.409 ± 0.300*
	5th day	2.283 ± 0.339	3.102 ± 0.361	2.932 ± 0.363	4.542 ± 0.278*
Root					
Growth (cm)	1st day	10.05 ± 0.335	10.06 ± 0.330	8.95 ± 0.384	8.27 ± 0.524
	5th day	12.47 ± 0.398	12.63 ± 0.369	10.18 ± 0.497	9.03 ± 0.471*
Fresh mass (g)	1st day	0.021 ± 0.0031	0.021 ± 0.0033	0.018 ± 0.004	0.017 ± 0.0034
	5th day	0.036 ± 0.0033	0.037 ± 0.032	0.025 ± 0.0032*	0.020 ± 0.0036*
Cu [$\mu g\ g^{-1}$ (f.m.)]	1st day	0.090 ± 0.022	0.384 ± 0.066	1.522 ± 0.106*	4.863 ± 0.104*
	5th day	0.106 ± 0.053	14.60 ± 0.505*	24.30 ± 0.714*	38.56 ± 0.688*
Ascorbate [$\mu mol\ g^{-1}$ (f.m.)]	1st day	583.79 ± 53.98	577.04 ± 53.36	615.66 ± 50.02	679.45 ± 46.22
	5th day	539.80 ± 50.86	592.81 ± 52.05	646.30 ± 53.30	698 ± 51.16
Glutathione [$\mu mol\ g^{-1}$ (f.m.)]	1st day	3686.68 ± 192.69	4629.29 ± 203.54*	5095.24 ± 199.84*	5226.48 ± 224.37*
	5th day	3889.27 ± 208.74	4951.57 ± 198.19*	5654.88 ± 213.81*	5999.17 ± 216.81*
Proline [$\mu mol\ g^{-1}$ (f.m.)]	1st day	2.233 ± 0.345	2.660 ± 0.420	3.457 ± 0.528	4.699 ± 0.321*
	5th day	2.619 ± 0.358	2.860 ± 0.420	3.854 ± 0.402	5.408 ± 0.548*

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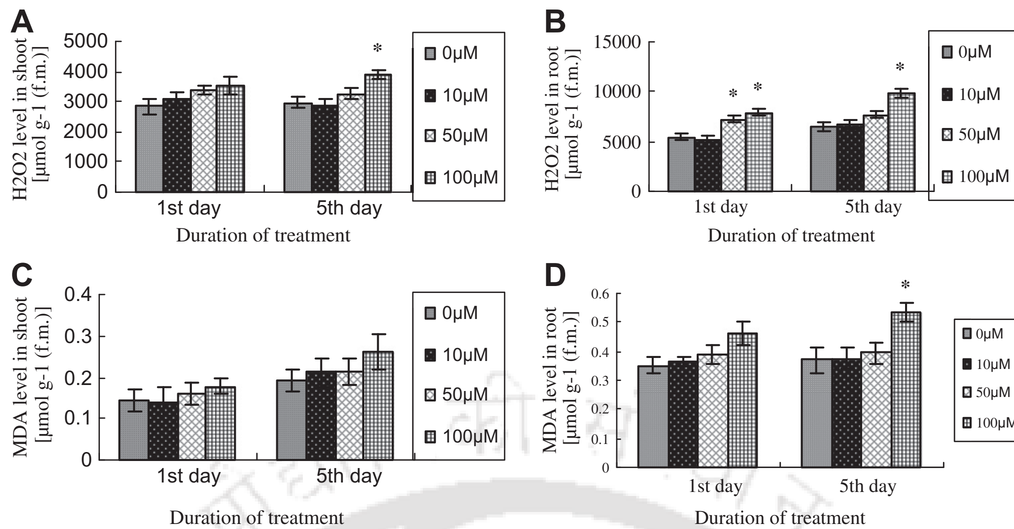

Fig. 1. Effect of different concentrations of Cu on contents of MDA in shoot (A), root (B) and H_2O_2 in shoot (C) and root (D). Data presented are mean ± SE ($n = 3$). *Significant mean difference from control at $P = 0.05$ in multiple comparison by LSD test.

pattern of enhancement in the proline content was recorded after 5th day where maximum content was recorded at 100 µM Cu by 98.95% in shoot and 106.49% in root over control.

2.6. Antioxidant enzymes activities

The antioxidant enzymes are important components in preventing oxidative stress in plants by scavenging free radicals and peroxides with the elevation of their activities when exposed to stressful conditions. Almost all the antioxidant enzymes have been greatly activated under stress condition.

In the present study, it is found that SOD activity increases by 62.85% and 64.58% at 100 µM Cu with respect to control in shoot and root respectively after 1st day recorded exposed to 100 µM Cu. Results of the isoenzyme patterns suggested that four isoenzyme bands (i, ii, iii and iv) were detected in the shoot (Fig. 2B) where band iv showed moderate expression at 10 µM Cu as compared to the expression under higher Cu concentrations. In root, three isoenzyme bands (i, ii and iii) were found and SOD iii was detected only at the higher concentration of Cu, 50 µM and 100 µM (Fig. 2D). Results showed the levels of SOD induced in response to copper stress however they differ in different copper concentration.

GPX activity has been increased in both shoot and root with an increase in Cu levels and duration of treatment (Fig. 3A, B). After 5th day of treatment, higher activity of GPX was observed where 19.52%, 33.81% and 68.49% in shoot and 22.83%, 43.47% and 80.46% in root increased respectively at 10, 50 and 100 µM Cu comparing to the control. The activity was higher in root than shoot. GR activity was increased in all the treated plant where significant increase was observed at the higher concentration of Cu after 5th day of treatment. Treatment with 100 µM increased the GR activity by 47.74% in shoot (Fig. 3C) and by 66.04% in root (Fig. 3D) over control. In this enzyme activity also root showed greater activity than shoot. The activity of APX enzyme increased progressively in a concentration and time dependent manner in both shoot and root. The activity of this enzyme was dramatically increased at 50 and 100 µM Cu after 5th day of treatment with 42.77% and 69.26% in shoot (Fig. 3E) and 48.44% and 69.26% in root (Fig. 3F) over the control respectively. In this study, CAT activity of the treated plants was almost equal with the control in both shoot and root in all the experimental conditions.

3. Discussion

3.1. Changes of growth and copper uptake

The decrease in growth of rice plant when raised under increasing level of Cu suggested Cu induced toxicity at elevated concentration and the impact was found more in root than shoot. The greater sensitivity of root to Cu toxicity might be due to the higher Cu content in root as maximum accumulation of Cu in rice root was observed. Similar observation was reported in *Elsholtzia splendens* [9] under Cu treatment. On the basis of this result, Cu toxicity is directly correlated with the accumulation of Cu in the plant. Greater Cu content in root than shoot indicates adoption of exclusion mechanism to tolerate the toxicity in which the roots accumulate the metals preventing its subsequent transport to the shoots [3]. Our results reconfirmed the earlier report showing correlation of Cu tolerance and its greater accumulation in root with poor translocation to shoot in Cu-tolerant plants [10]. A slight increase in Cu concentration from the optimal requirement caused toxicity to most of the plants. Even 4 µM Cu drastically inhibited the root length of *Paspalum distichum* and *Cynodon dactylon* [11]. However, in our study not much significant changes in root and shoot length and biomass were recorded even at 50 µM Cu concentrations during early stages of Cu stress suggesting the Cu tolerance of MSE-9 rice variety under study.

3.2. Hydrogen peroxide level

In the current finding increase in Cu concentration led to the increase in H_2O_2 production. The root showed higher H_2O_2 content than shoot indicating root is the primary site for ROS production. It might be due to the higher Cu content in root as Cu induced ROS production in cells. Copper and other heavy metals like Cr, Zn and Ni have been reported previously to produce ROS like $O_2^{\bullet -}$, H_2O_2 and OH• which in turn induce oxidative damage and cell death in plants [12–14].

3.3. Lipid peroxidation

As MDA is a product of lipid peroxidation, the elevation in MDA content clearly reflects cell wall damage. Our results indicate that excess Cu increases oxidative stress as is evident from increased

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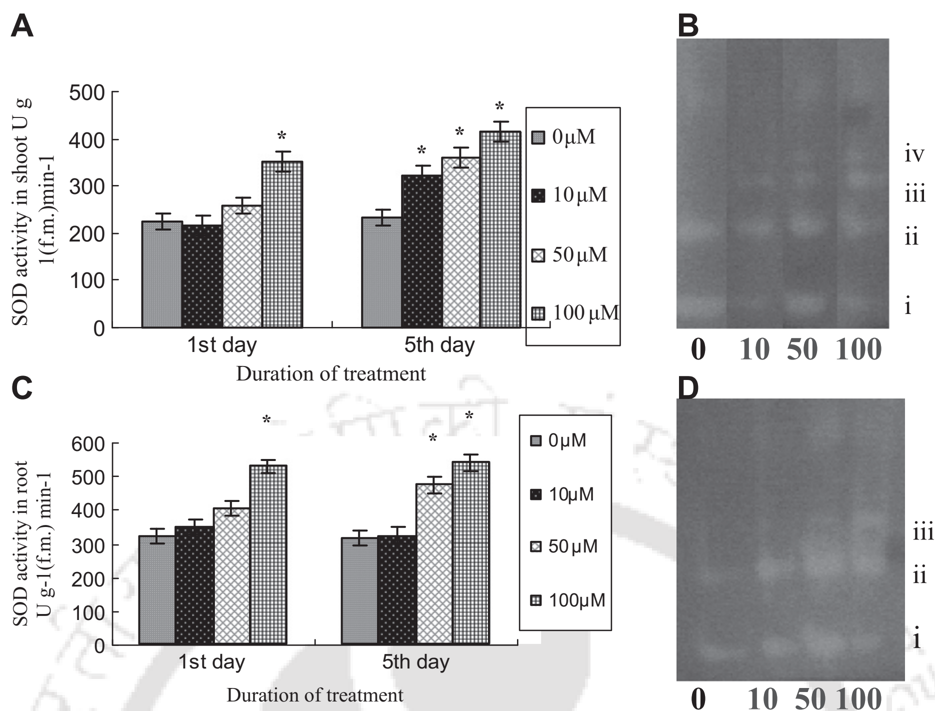

Fig. 2. Effect of Cu on the activities and isoenzyme patterns of SOD in shoot (A and B) and root (C and D). B and D showed the isoenzyme activity in shoot and root respectively after 5th day of treatment under different concentrations of Cu, 0 µM, 10 µM, 50 µM and 100 µM. Data presented are mean ± SE ($n = 3$). *Significant mean differences from control at $P = 0.05$ in multiple comparison by LSD test.

lipid peroxidation. It is accordance with the previous findings [15] that MDA accumulated greatly after the exposure of Cu and cell membrane is the primary site of Cu toxicity. It might be due to the overproduction of ROS under Cu stress which is highly destructive to cell membrane.

3.4. Ascorbate and glutathione contents

The present study showed that ASH and GSH contents increase in all the concentrations of Cu of this experiment. Significant increase in GSH content in all the treated plant in root reveals increase in Cu tolerance as GSH is involved in keeping the ROS in balance. Greater enhancement in GSH content in root might be due to combat the damage induced by the overproduction of H_2O_2 in root. Pastori and Foyer [16] reported that GSH, as an antioxidant, helped to withstand oxidative stress in transgenic lines of tobacco. Increased in the GSH content in all the Cu treated plant also reflects biosynthesis of phytochelatins (PCs), as GSH is a precursor for PCs [17] which may involve in detoxification and tolerance by chelating the metals [5]. ASH is also one of the main antioxidant that contributes to resistance against oxidative stress. It influences many enzyme activities and minimizes the damage caused by oxidative process through synergic function with other antioxidants [18]. Increment in ASH content under Cu stress indicates its involvement in the Cu tolerance mechanism.

3.5. Proline content

The result suggested that accumulation of proline responses to Cu and it varies with respect to the toxicity caused by Cu treatment. Accumulation of proline in tissue/organs of plants under heavy metal pollution has been reviewed by Alia and Pardha Saradhi [19]. Proline accumulation prevents membrane distortion and acts as a hydroxyl radical scavenger. Mehta and Gaur [20] also noted a protective role of proline in mitigating metal-induced lipid peroxidation in *Chlorella vulgaris*. Thus greater accumulation of proline under high Cu level of the present study suggested the protective role of proline to the plant to survive Cu stress.

3.6. Antioxidant enzymes activities

SOD constitutes the first line of defense against ROS. SOD is a metalloenzyme that dismutate superoxide radical to H_2O_2 and oxygen. Increase in SOD activities in stressed plants was indicative of enhanced $O_2^{\bullet-}$ production and oxidative stress tolerance and has been proposed as an important enzyme for plant stress tolerance. The results of the current study showed constant detoxification of the $O_2^{\bullet-}$ that might have been generated. Recent report also showed increase in SOD activity under Cu stress in *Elsholtzia haichowensis* [21].

In this study, GPX appeared to be an important enzyme in overcoming Cu imposed oxidative stress as there has been a considerable increase in GPX activities in the stressed plants. This was in accordance with the previous report in *Pavlova viridis* [22]. GPX is the antioxidant enzyme capable of removing H_2O_2, neutralizing or scavenging free radicals. Significant increase in GPX activity in the present study reflects its vital role in scavenging H_2O_2 produced by the Cu stress.

Increased GR activity in the Cu treated plant could be an acclimatization step against Cu stress as it is an important enzyme essential for the appropriate functioning of the antioxidant system in plants. It involved in the enzymatic detoxification of ROS and contributes to the maintenance of a higher GSH to GSSG ratio. Similar finding has been reported in *B. juncea* under Cd stress [23].

Increase in APX activities both in shoot and root of the rice plant when were exposed to Cu stress showed its role in the adaptation process against Cu induced oxidative stress. Enhancement of APX activity under Cu stress has been previously reported [24]. APX

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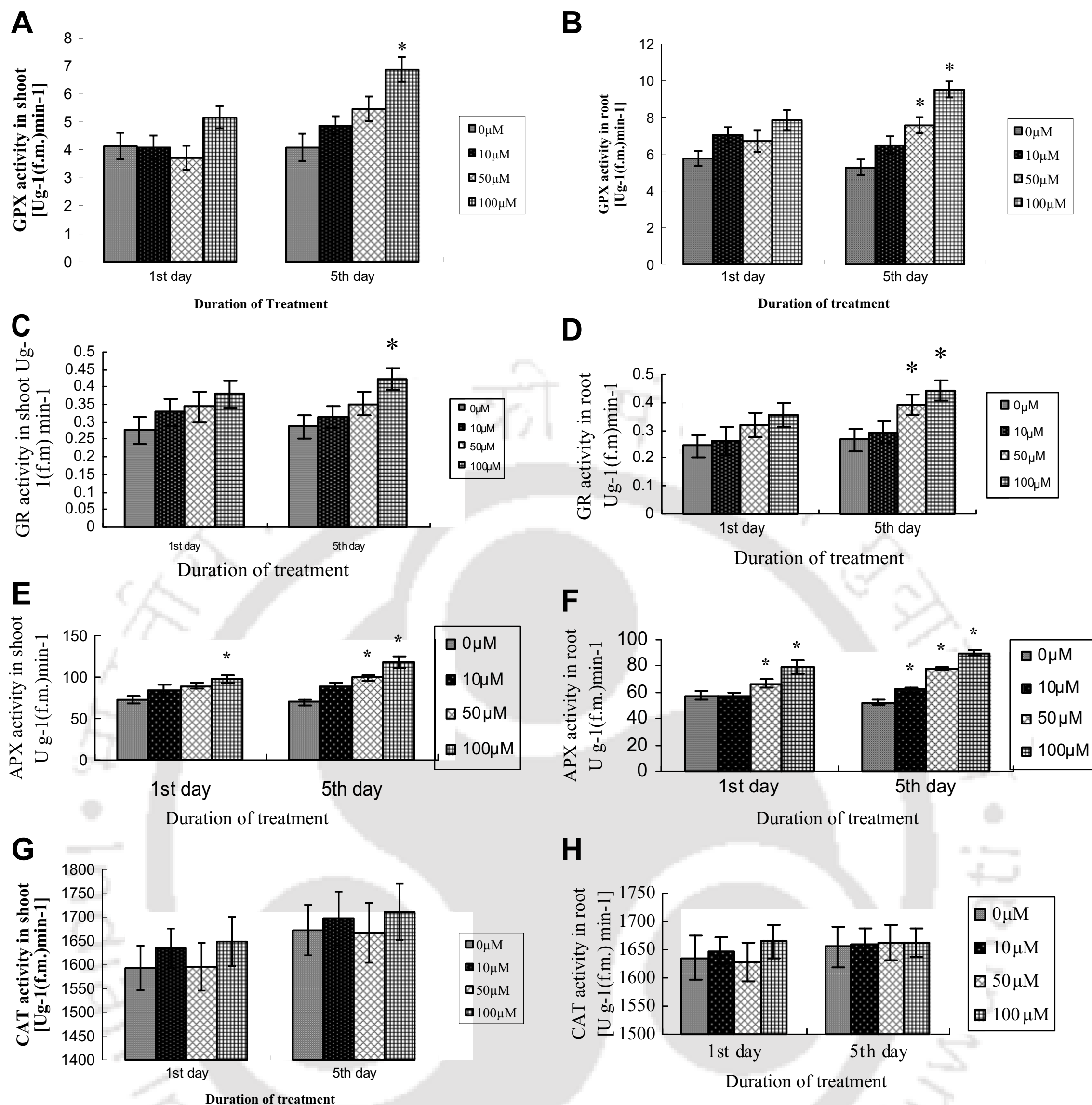

Fig. 3. Effect of different concentration of Cu on the activities of GPX (A and B), GR (C and D), APX (E and F) and CAT (G and H) in shoot and root respectively after 1st and 5th day of treatment. Data presented are mean ± SE ($n = 3$). *Significant mean difference from control at $P = 0.05$ in multiple comparison by LSD test.

detoxify H_2O_2 using ascorbate for reduction. It is one of the major components of ASH–GSH cycle where it functions to prevent the accumulation of toxic levels of H_2O_2 in photosynthetic organisms. Thus increase in APX activity with the higher content of GSH and ASH in the Cu treated rice plant of this study might relate with proper functioning of ASH–GSH cycle that detoxifies H_2O_2 thereby preventing from the damage of Cu toxicity. This is in accordance with the findings of Drazkiewicz et al. [25], where ASH–GSH cycle plays an important role in reducing the toxic effect of Cu.

The present study showed that CAT activity did not show significant variation both in shoot and root of the rice plant. CAT is an enzyme which can eliminate H_2O_2 from the plant by degrading it to water and O_2. No definite variation in the CAT activity of the present finding suggests CAT appears not to be required for the elimination of H_2O_2 in the Cu stress rice plant. It might be due to removal of H_2O_2 is carried out by APX and GPX so upgrade in the CAT activity is not required. It has also been reported that CAT activity was not affected by Cu treatment [26]. The response of CAT activity differs broadly depending on the plant species and the metals. Decline in CAT activity was reported in *B. juncea* L. under Cu toxicity [3] whereas enhancement of CAT activity was observed in various plants under several metal toxicity including Cu [27] and Cd [28]. The enhancement in the activities of the antioxidant enzymes is responsible for the scavenging of ROS showing adaptive response of rice plant against Cu stress.

Regarding the present findings, excess Cu induced oxidative stress in rice plant enhancing the ROS production and increased lipid peroxidation. Root suffered more damage than shoot. Stimulation of antioxidant enzymes and antioxidant contents reflects the ability of the rice plant to withstand the Cu induced oxidative stress. Proline accumulation also appears to be an additional defense against Cu oxidative stress.

4. Materials and methods

4.1. Plant cultivation and treatments

Viable rice seed (MSE-9) variety was procured from Regional Agricultural Research Station (AAU), Akbarpur, Karimganj, Silchar. The seeds were surface sterilized with 0.1% mercuric chloride

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($HgCl_2$) solution, rinsed with distilled water and set for germination in clean petri dishes with wetted filter papers at 30 °C in dark. Uniformly germinated seeds were selected and were transferred to the plastic pots containing Hoagland nutrient medium (pH = 6.2). The seedlings were grown in a growth chamber under continuous white light provided with cool, fluorescent white tubes (Philips 20W TLD, India). Copper treatment in the form of $CuSO_4$ of different concentration 10, 50 and 100 μM were given on the 5th day of transferred. Plants were harvested after 1st and 5th day of treatments for various analysis.

4.2. Growth and copper content analysis

Growth of the plant was determined by measuring the length and fresh mass of shoot and root of the plant. The shoot and root were oven dried for 72 h at 80 °C and acid digestion was done as per the method of Humpries [29]. The dry mass (0.1 g) was digested with 5 ml aquaregia in a beaker until dried and made the volume upto 20 ml by adding deionized water and filtered. The total Cu content was measured using atomic absorption spectrometer (*Perkin–Elmer 3110*, Germany).

4.3. Determination of hydrogen peroxide level

The total H_2O_2 content was estimated as per the method of Sagisaka [30]. H_2O_2 was extracted by homogenizing 200 mg of tissue with 5 ml 5% TCA. The homogenate was centrifuged at 12,500 *g* for 10 min. 40 μl of 50% TCA, 20 μl of KSCN and 40 μl of ferrous ammonium sulphate were added to the supernatant to determine H_2O_2 levels. The absorbancy was measured at 480 nm.

4.4. Lipid peroxidation determination

The level of lipid peroxidation is estimated by the method of Heath and Packer [31] in term of MDA content determined by thiobarbituric acid (TBA) reaction. 200 mg of the tissue was homogenized with 5 ml 0.25% TBA. The homogenate was boiled for 30 min at 95 °C and centrifuged at 10,000 *g* for 10 min. The absorbance of the supernatant was recorded at 532 nm and corrected by substracting absorbance at 600 nm.

4.5. Extraction and assay of non-enzymic antioxidant, glutathione and ascorbate

Total glutathione and ascorbate contents were determined as per the method Griffith [32] and Oser [33]. Plant was homogenized with 5 ml phosphoric acid and centrifuged at 17,000 *g* for 15 min at 4 °C. The supernatant was collected and used for assay preparation.

The reaction mixture for ascorbate content contained 2% sodium molybdate, 0.1 N H_2SO_4, 1.5 N Na_2HPO_4 and plant extract. After incubation at 60 °C for 40 min again centrifuged at 3000 *g*. Absorbancy was measured at 660 nm. Glutathione assay mixture contained plant extract, 0.1 M phosphate buffer (pH 6.8), DTNB (5,5-dithiobis-2-nitrobenzoic acid) and the absorbancy was recorded at 412 nm.

4.6. Determination of proline content

Proline concentration was determined following the method of Bates et al. [34]. Plant tissue (0.5 g) was homogenized with 5 ml of sulfosalicylic acid (3%) using mortar and pestle and filtered through Whatman No. 1 filter paper. The volume of filtrate was made up to 10 ml with sulfosalicylic acid and 2.0 ml of filtrate was incubated with 2.0 ml glacial acetic acid and 2.0 ml ninhydrin reagent and boiled in a water bath at 100 °C for 30 min. After cooling the reaction mixture, 6.0 ml of toluene was added, mixed in a vortex mixer and absorbance was read at 570 nm.

4.7. Antioxidant enzymes extraction and assay

For the extraction and estimation of enzymes, plant was homogenized in 0.1 M potassium phosphate buffer, pH 6.8 in a chilled pestle and mortar. The homogenate was centrifuged at 17,000 *g* for 15 min at 4 °C and the supernatant was used for the assay of enzyme activity of SOD, GPX, GR and CAT. For the extraction of APX, 1 mM ascorbate was added to the extraction buffer to restore the activeness of the enzyme.

The assay of SOD was carried out by the method of Gianopolitis and Reis [35]. The assay mixture for SOD (1.15.1.1) contained 79.2 mM Tris–HCl buffer (pH 6.8), containing 0.12 mM EDTA and 10.8 mM tetraethylene diamine, bovine serum albumin (0.0033%), 6 mM nitroblue tetrazolium (NBT), 600 μM riboflavin in 5 mM KOH and 0.2 ml enzyme extract. Reaction was initiated by placing the glass test tubes in between two fluorescent tubes (*Philips 20W*). By switching the light on and off, the reaction was started and terminated, respectively. The increase in absorbance due to formazan formation was read at 560 nm. The activity was expressed as ΔA_{560} (g^{-1} (fr.wt.) min^{-1}).

GPX activity was assayed according to Chance and Maehly [36]. The reaction mixture for GPX (1.11.1.7) contained 2.1 ml of 0.1 M phosphate buffer (pH 6.8), 0.3 ml of 1% guaicol, 0.3 ml of 1% H_2O_2, 0.3 ml of enzyme extract and incubated for 5 min. Absorbancy was taken at 470 nm (extinction co-efficient, $\varepsilon = 26.6$). The activity was expressed as ΔA_{470} (g^{-1} (fr.wt.) min^{-1}).

Glutathione reductase (GR) was assayed by the method of Smith et al. [37]. The reaction mixture of GR (1.6.4.2) contained 1.0 ml of 0.2 M potassium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.5 ml of 3 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) in 0.01 M potassium phosphate buffer (pH 7.5), 0.1 ml of 2 mM NADPH, 0.1 ml enzyme extract and distilled water to make up a final volume of 2.9 ml. Reaction was initiated by adding 0.1 ml of 2 mM GSSG (oxidized glutathione). The increase in absorbance at 412 nm was recorded at 25 °C over a period of 5 min spectrophotometrically. The activity is expressed as absorbance change (ΔA_{412}) (g fresh $mass^{-1}$ s^{-1}).

CAT activity was assayed according to Chance and Maehly [36]. The reaction mixture for CAT (1.11.1.6) comprised of 3.0 ml of 0.1 M phosphate buffer (pH 6.8), 1.0 ml of (30 mM) H_2O_2 and 1.0 ml of enzyme extract. The reaction was stopped by adding 10 ml of 2% H_2SO_4 after 1 min incubation at 20 °C and the absorbancy of the reaction mixture was recorded at 240 nm (extinction co-efficient, $\varepsilon = 43.6$). The CAT activity was expressed as unit min^{-1} g (fr.wt).

For APX (1.11.1.11) extraction, according to Nakano and Asada [38], the tissue sample was homogenized with 0.1 M phosphate buffer (pH 6.8) containing 1 mM ascorbate, centrifuged at 17,000 rpm for 15 min at 4 °C. The supernatant was used as enzyme extract for assay mixture. The assay mixture was prepared with 10 mM H_2O_2, 0.5 mM ascorbate, 0.1 M phosphate buffer (pH 6.8), enzyme extract. The absorbancy was taken at 290 nm. With extinction co-efficient 2.8 mM^{-1} cm^{-1}, the APX activity was expressed as ΔA_{290} (g^{-1} (fr.wt.) min^{-1}).

Protein concentrations were estimated according to Bradford [39] using bovine serum albumin as standard.

4.8. Gel electrophoresis

The isoenzyme of SOD was separated by non-denatured electrophoresis on 10% polyacrylamide vertical slab gels under constant current (120 mA) at 4 °C. SOD activity was detected by photochemical staining after electrophoresis according to Ádám et al. [40]. Gel was incubated in a solution containing 50 mM sodium

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phosphate buffer (pH 7.8), 0.1 mM EDTA, 3 mM riboflavin and 0.25 mM nitroblue tetrazolium chloride in the dark for 20 min at 25 °C. After rinsing with distilled water, gels were illuminated under light until the colorless bands were clear against a blue/purple background.

4.9. Statistical analysis

All data obtained were subjected to one-way analyses of variance (ANOVA) and LSD test was used for comparison between mean of treatments. Each bar represents mean ± standard error of three experiments. An asterisk indicates a significant mean difference from control at $P = 0.05$.

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