Studies on the induction of variation through *in vitro* culture in *Jatropha curcas* L.

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by

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Dedicated to my beloved family.....

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CERTIFICATE

This is to certify that the research work presented in this thesis entitled **"Studies on the induction of variation through *in vitro* culture in *Jatropha curcas* L."**, is based on the original research work carried out by Ms. Jikku Jose under my guidance and supervision in the Department of Biotechnology, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, and that no part thereof has been presented for the award of any other degree, diploma, associateship or other similar titles or recognition.

Dr. Padma Nambisan

CUSAT,

30-01-2013.

DECLARATION

I hereby declare that the work presented in this thesis entitled **"Studies on the induction of variation through *in vitro* culture in *Jatropha curcas* L."**, is based on original research work carried out by me at the Department of Biotechnology, Cochin University of Science and Technology, Cochin under the guidance of Dr. Padma Nambisan, Associate Professor, Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, India and that no part thereof has been presented for the award of any degree or diploma, associateship or other similar title or recognition.

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ABBREVIATIONS

%	Percentage
~	Approximately
<	Less than
>	Greater than
≥	Greater than or equal to
°C	degree Celsius
µg/mL	Microgram/ milliliter
µL	Microlitre
µM	Micromolar
µm	micrometre
2, 4-D	2,4-Dichlorophenoxyacetic acid
AFLP	Amplified fragment length polymorphism
ANOVA	Analysis of variance
AS	Adenine sulphate
ASSR	Anchored simple sequence repeats
ATP	Adenine triphosphate
BA	benzyladenine
BAP	6-Benzylaminopurine
bp	Base pair
C	Control
CA	Chlorogenic acid
cm	Centimeter
Conc.	Concentration
CRD	Completely randomized design
CTAB	Cetyl trimethylammonium bromide
$CuSO_4$	Copper sulphate

CycD3	Cyclin D3
DAMD	Direct amplification of minisatellite Dna markers
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
etc.	Et cetera
ft	Feet
g	Gram
GA_3	Gibberellic acid
GC	Guanine- Cytosine
GI-M	Gap 1 to Mitosis phase
h	hour
ha	Hectare
HCl	Hydrochloric acid
HIV	Human immuno virus
HPLC	High pressure liquid chromatography
HPTLC	High pressure thin layer chromatography
i.e.	That is
I.G.P	*In vitro* generated plants
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSR	Inter-simple sequence repeats
ITS	Internal transcribed spacer
JNTBGRI	Jawaharlal Nehru Tropical Botanical Garden Research institute
KCl	Potassium chloride
kg	Kilogram
kg sq.cm	Kilogram square centimetre

Kn	Kinetin
L	Leaf
M	Molar
m	metre
M.P	Mother plant
mg/L	Milligram/litre
$MgCl_2$	Magnesium chloride
MI	Mitotic index
MIC	Minimum inhibitoy concentration
min	Minute
MIP	Methylation induced premeiotically
ml	Milliliter
mm	Millimeter
mRNA	Messenger ribonucleic acid
MS	Murashige and Skoog
MSI	Mitotic stage index
N	Normal
NAA	1-naphthaleneacetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCIM	National Collection of Industrial Microorganisms
ng	nanogram
No.	number
P	Petiole
p	probability
PCR	Polymerase chain reaction
PGR	plant growth regulator
pH	Potential of hydrogen

PHYLIP	PHYLogeny Inference Package
POD	Peroxidase
PPO	Polyphenol oxidase
R	Root
RAPD	Random amplified polymorphic DNA
rDNA	Recombinant deoxyribonucleic acid
Rf	Retention factor
RIP	Repeat induced - point mutation
rpm	Rotation per minute
S	Stem
S. No.	Serial number
SD	Standard deviation
SE	Standard error
SPSS	Statistical package of social science
SSR	Single sequence repeats
t.	tonn
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TDZ	Thidiazuron
TE	Tris EDTA
TEs	Transposable elements
TLC	Thin layer chromatography
U.S.DOE	United States department of energy
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultra violet
V	Volt
v/v	volume per volume
v/v/v	Volume per volume per volume

w/v	Weight per volume
WHO	World Health Organization

CONTENTS

CHAPTER I

INTRODUCTION

Jatropha is a large genus comprising more than 170 species. The commonly occurring species in India are *J. curcas, J. glandulifera, J. gossypifolia, J. multifida, J. nana, J. panduraefolia, J. villosa, and J. podagrica.* Most of these species are ornamental, except for *J. curcas* and *J. glandulifera*, which are oil-yielding species (Swarup, 2004). *Jatropha curcas* L. is native to South America. It is distributed naturally in the tropical Americas and became naturalized in many parts of the tropical and subtropical regions of the world (Heller, 1996; Gohil and Pandya, 2008). Even though Jatropha has its natural distribution in the north-eastern parts of South America, it is now found abundantly in many tropical and sub-tropical regions throughout Africa and Asia (Jongschaap *et al.*, 2007). Jatropha was probably disseminated by Portuguese seafarers via the Cape Verde Islands and Guinea Bissau to other countries in Africa and Asia (Heller, 1996). The plants are highly drought tolerant and are suitable for preventing soil erosion and shifting of sand dunes. *J. curcas* is a multipurpose plant valued for its medicinal properties, resistance to various stresses and its use as an oilseed crop (Heller, 1996; Openshaw, 2000).

I. 1. BOTANICAL DESCRIPTION OF *JATROPHA CURCAS* LINN.

Jatropha curcas L. (purge nut, physic nut) belonging to the family Euphorbiaceae, is a common perennial shrub with many attributes. The genus belongs to the tribe Jatropheae. Euphorbiaceae is an ancient and diverse family in the large rosid order Malpighiales and in addition to Jatropha it includes familiar members such as rubber, cassava, castor bean, poinsettia and leafy spurge (Wurdack, 2008). Classification of the genus Jatropha is depicted in Table 1.1.

Table 1.1: Classification of the genus *Jatropha*.

Kingdom	**Plantae**
Subkingdom	**Tracheobionta**
Super division	**Spermatophyte**
Division	**Magnoliophyta**
Class	**Magnoliopsida**
Subclass	**Rosidae**
Order	**Euphorbiales**
Family	**Euphorbiaceae**
Genus	***Jatropha***

J. curcas is a small tree or large shrub, which can reach a height of 3 to 5 m, and under favourable conditions, can attain a height of 8 to 10 m. It has green leaves with a length and width of 6 to 15 cm, with 5 to 7 shallow lobes. The leaves are arranged alternately. The plant shows articulated growth, with amorphological discontinuity at each increment. Leaves five to seven lobed, hypostomic and stomata are of paracytic (Rubiaceous) type (Kumar and Sharma, 2008). The trees are deciduous, shedding the leaves in dry season. Flowering occurs during the wet season. Petiole is circular in cross section. The epidermis is thin and less conspicuous. Outer ground tissue is collenchymatous and inner is parenchymatous. The vascular system consists of several (more than 15) discrete vascular bundles forming a medullated ring. The vascular bundles are collateral, with separate rows of xylem and small patches of phloem. Circular, thick walled laticifers are distributed in the outer cortex of petiole (Uthayakumari and Sumathy, 2011). Normally, five roots are formed from seeds: one tap root and four lateral roots. Plants from cuttings develop only lateral roots. Inflorescences are formed terminally on branches. The plant is monoecious with male and female flowers

produced in the same raceme (Raju and Ezradanam, 2002) and flowers are unisexual (Dehgan and Webster, 1979). Pollination is by insects. On the basis of available information Ambrosi *et al.* (2009) reported that, 68% of seeds are set through amphimixis, mainly by outcrossing (enthomophylous pollination), even if the species is self compatible and hence selfing is also possible. The average degree of apomixis is 32%. Agamospermy (*i.e.* embryo sacs and embryos produced in ovules without meiotic reduction or egg cell fertilization), as a mode of asexual reproduction through seed, leads to clonality. Nevertheless, the species seems to show a tendency to promote xenogamy (*i.e.* union of genetically unrelated organisms) and to minimize geitonogamy (*i.e.* the pollination of a flower with the pollen from another flower on the same plant), mechanisms that increase diversity. Luo *et al.* (2007) studied the reproduction characteristics of *J. curcas* in Yuanjiang County and concluded that the plant produces flowers in dichasial inflorescences. After pollination, a trilocular ellipsoidal fruit is formed. The exocarp remains fleshy until the seeds are mature. The seeds are black and on average 18 mm long and 10 mm wide in ripe Jatropha fruits (Singh, 1970).

I.2. ECONOMIC IMPORTANCE OF *J. CURCAS*

In the plant kingdom, Euphorbiaceae family members are rich sources of pharmacological products, oil, dyes, rubber, timber, furniture, agricultural implements, aesthetic items, edible tubers, or are ornamental plants. *Jatropha curcas* plant itself is a reactor for production of many phytochemicals, such as alkaloids, terpenes, lignins, essential amino acids, and cyclic peptides and can potentially be used as a source of oil, animal feed or medicinal preparations and produces cosmetics, soap, anti cancer medicines and pesticides. The seed oil is found to be efficient as biodiesel. Almost all the parts of the plant are economically important (Figure 1.1).

Figure 1. 1: Various uses of *Jatropha curcas*

1.2.1. Use of *J. curcas* in folk medicine

For centuries, plants have proved to be a source of novel drug compounds, and plant derived medicines have contributed to human health and well being. Natural products, either as pure compounds or as standardized plant extracts, provide opportunities for new drug leads because of the chemical diversity. Traditional medicine using plant extracts continues to provide health coverage for over 80% of the world's population, especially in the developing world (WHO, 2002). Therefore, researchers are increasingly turning their attention to

folk medicine and looking for new leads to develop better drugs against microbial infections (Benkeblia *et al.*, 2004).

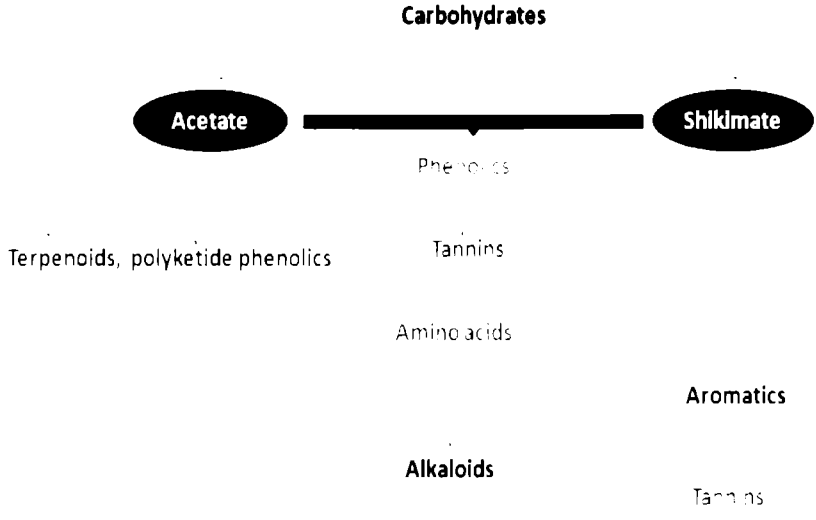


Figure 1.2: Secondary metabolite production in plants

Among the compounds derived from plants (phytochemicals), secondary metabolites play a crucial role as pharmaceuticals due to their structural diversity. They are derived from primary metabolites as depicted in Figure 1.2. Each plant family, genus, and species produces a characteristic mixture of these chemicals, and they can sometimes be used as taxonomic characters in classifying plants. Single group of secondary metabolite dominates within a given taxon. In plants, secondary metabolites apparently function as defense (against herbivores, microbes, viruses or competing plants) and signal compounds (to attract pollinating or seed dispersing animals). Secondary metabolites can be classified on the basis of chemical structure (having carbon rings, or containing sugar), composition (containing nitrogen or not), their solubility in various solvents, or pathways by which they are synthesized (for example, phenylpropanoid pathway which produces tannins).

Some secondary metabolites concomitantly carry out physiological functions, for example, alkaloids and peptides (lectins, protease inhibitors) can serve as mobile and toxic nitrogen transport and storage compounds, or phenolics such as flavonoids, can function as UV-protectants (Wink, 1993).

The genus name Jatropha derives from the Greek *jatros* (physician), *trophe* (food), which implies its medicinal uses. Pharmacological applications of *Jatropha curcas* are well-known but much of the information is empirical and lacking scientific validation (Oskoueian *et al.*, 2011). The extracts of many *Jatropha* species including *Jatropha curcas* displayed potent cytotoxic, antitumour, and antimicrobial activities in different assays (Singh *et al.*, 2012). *J. curcas* is also efficacious in treatment of dropsy, sciatica and paralysis. The active component in its latex jatrophone has shown anti-cancer properties (Kosasi *et al.*, 1989). Curcacycline A displays moderate inhibition of classical pathway of human complement activity and proliferation of human T-cells (Berg *et al.*, 1995). Curcacycline B (Auvin *et al.*, 1997), pohlianin A has antifungal and antimalarial activity (Auvin *et al.*, 1999) and jatrophidin I has antifungal activity (Altei *et al.*, 2008). Fagbemo-Beyioku *et al.* (1998) investigated and reported the anti-parasitic activity of the sap and crushed leaves of *J. curcas*. Polymers have been derived from Jatropha by scientists at the Central Salt Marine Chemicals and Research Institute in Bhavnagar, India, with putative multiple uses such as the development of artificial blood vessels (Nair and Avinash, 2010). The drug obtained from *J. curcas* is termed as 'Dravanti' and is reported to be bitter, pungent and astringent in taste (Gupta, 1985).

1.2.2. Use of *J. curcas* as a source of bio-fuel

The increasing fuel demand and hike in price adversely affect the day to day life of common people. Diminishing petroleum reserves and hazardous pollutants from petroleum fuelled engines necessitates a search for an alternative

renewable fuel source. Since India imports 70% of its fuel, there exists a ready market for biofuels and 10% biodiesel blends (Kumar and Sharma, 2008).

There is growing interest in the use of *J. curcas* oil to alleviate the energy crisis. *J. curcas* oil is relatively simple to convert to biodiesel by chemical or biological trans esterification (Modi *et al.*, 2007). In addition to the low production cost, *J. curcas* biofuel has been reported to be non-toxic, clean and eco-friendly (Jha *et al.*, 2007). Jatropha biofuel contains more oxygen, with a higher cetane value increasing the combustion quality. It does not contribute to a rise in the level of carbon dioxide in the atmosphere (Korbitz, 1999; Beet *et al.*, 2002; Sims, 2001).

J. curcas produces large quantity of oil-seed within 2 to 3 years after planting. The species is highly open pollinated and can be propagated from seed or cuttings of stem or branch. It starts flowering and fruiting one year after planting. The seeds mature about 3-4 months after flowering. Economic yield of seeds starts after 3 years and continues for a period of 50 years (Aker, 1997).

The seed weighs about 0.75 g and the kernel represents about 65% of the seed mass. Reports on the chemical composition of the kernel indicate protein content of 27-32% and lipid content of 58- 60% (Liberalino *et al.*, 1988; Aderibigbe *et al.*, 1997). The seed weight per 1000 seeds is about 727 g, and on an average, there are 1375 seeds/kg (Kamal *et al.*, 2011). The seeds contain semi-dry oil (Gubitz *et al.*, 1999). The oil content is 25-30% in the seeds and 50-60% in the kernel. Saturated fatty acids constitute 20% of this, the rest being unsaturated fatty acids. Oleic acid is the most abundant (44.8%) followed by linoleic acid (34.0%), palmitic acid (12.8%) and stearic acid (7.3%). Seed yield and oil content are the most desirable traits in a species like *Jatropha.*

Biodiesel (which is alkyl esters of fatty acids) has been proposed as an alternative fuel source. There are many sources of biodiesel such as vegetable oils including soya bean, sunflower oil, waste food oil, animal fats, etc. Since these are competing as food source, the "Food or fuel" dilemma encouraged the

development of non edible biofuel crops. Among the non-edible oleaginous plants that have been described in the literature as sources of alkyl ester (biodiesel) are: neem (*Azadirachta indica*) (Meher *et al.*, 2005), karanja (*Pongamia pinnata*) (Raheman and Phadatare, 2004; Modi *et al.*, 2007; Raheman and Ghadge, 2007; Sharma and Singh, 2008; Srivastava and Verma 2008), mahua (*Madhuca indica*) (Puhan *et al.*, 2005), undi (*Calophyllum inophyllum*) (Azam *et al.*, 2005; Banapurmath *et al.*, 2008), castor bean (*Ricinus communis*) (Goodrum and Geller, 2005; Scholz and da Silva, 2008), simarouba (*Simarouba glauca*) (Adjaye *et al.*, 1995; Azam *et al.*, 2005), Benoil tree (*Moringa oleifera*) Rashid *et al.*, 2008), Jojoba (*Simmondsia chinensis)* (Bouaid *et al.*, 2007; Canoira *et al.*, 2006), rubber tree (*Hevea brasiliensis*) (Ramadhas *et al.*, 2005), Chinese tallow tree (*Sapium sebiferum*) (Gao *et al.*, 2008), Babassu (*Attalea speciosa*) (Oliveira *et al.*, 1999), tucum (*Astrocaryum vulgare*) (Li *et al.*, 2008), Zanthoxylum (*Zanthoxylum bungeanum*) (Zhang and Jiang, 2008), sea mango (*Cerbera odollam* or *Cerbera manghas*) (Gaillard *et al.*, 2004) and *J. curcas* (Kumar and Sharma, 2008).

Jatropha can be a competitive feedstock when compared to soybeans and rape seed, which produce lower oil yields than Jatropha (U.S. DOE 2010; Figure 1.3).

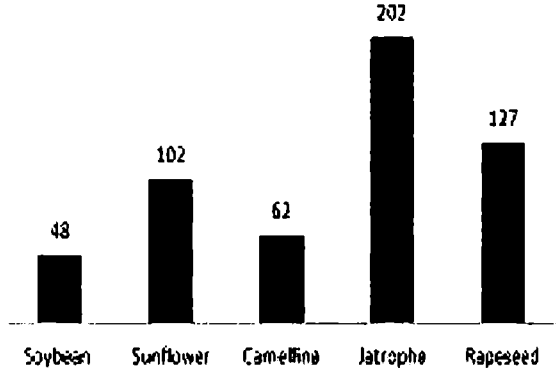


Figure 1.3: Comparison of oil yields from different biomass feedstock (Source: U.S. DOE 2010)

Besides biodiesel, *Jatropha* cultivation can make considerable impact in promoting organic farming through use of deoiled seed cake. Seed cake obtained after expelling the oil is rich in proteins (19%), carbohydrates (17%), nitrogen (6%), phosphorus (2.75%) and potassium (0.94%). Jatropha meal (fully defatted) has a crude protein content of between 53 and 63% and about 90% of this is present as true protein (Aderibigbe *et al.*, 1997).

I.3. PROPAGATION METHOD

Jatropha curcas seeds are genetically heterozygous as Jatropha sp. forms artificial and natural hybrid complexes readily and poses a problem to genetic fidelity (Prabakaran and Sujatha, 1999). Propagation through seed (sexual propagation) leads to genetic variability in terms of growth, biomass, seed yield, and oil content. Jatropha seeds are oily and cannot be stored for long- seeds older than 15 months show viability below 50 % (Kobilke, 1989). Many factors influence the establishment by direct seeding of Jatropha *curcas* such as, seeding depth, age and quality of seed, soil moisture content, quality of soil preparation (Heller, 1996). Due to its perennial nature, seed setting requires 2 to 3 years time. Seed production ranges from about 0.13 to 4.86 t. per acre (0.3–10.9 t. per ha) per year (Openshaw, 2000).

Vegetative propagation has been achieved by stem cutting, grafting, and budding as well as by air layering techniques. The optimal stem for cutting is 0.08 inches (2.0 mm) in diameter and 1.0 ft (30.4 cm) in length. Distal portion of the stock plants exhibit reduced rooting potential, while cuttings from the lower or juvenile regions of the plants generally maintain a higher rooting capacity than those from the upper regions (Hartmann and Kester, 1983). The stem pieces can be cut from the mother plant and planted at any time of the year. If it is planted during a dry period, irrigation is required. Direct planting by cutting decreased the time of production as compared to direct seeding or transplanting but showed a

lower longevity and a lower drought and disease resistance than those propagated by seeds (Heller, 1996). Plants produced from cuttings do not produce true taproots, rather, they produce pseudo taproots that may penetrate only one half to two thirds the depth of the soil compared to taproots produced on seed grown plants and hence are less drought tolerant (Heller, 1996). Therefore, propagation using cuttings is not the preferred method of most growers.

I.4. *IN VITRO* CULTURE

Plant tissue culture is a group of techniques for the maintenance and growth of plant cells or organs which can help in the propagation, genetic improvement and conservation of elite plant species. Any part of the plant such as, leaf, meristem, stem, petiole, anther, ovule, or pollen, can be used for the *in vitro* culturing. By mass propagation through tissue culture, it is possible to make available in large scale valuable plant species throughout the year, independent of season. Production of bioactive compounds can be enhanced by *in vitro* cultures without sacrificing the whole plant.

Systems of plant regeneration can be categorized as direct and indirect (Mukherjee *et al.*, 2011). In direct method, adventitious shoot formation occurs without callus phase and in indirect method regeneration occurs through intermediary callus phase. Shoots can be derived either through differentiation of non-meristematic tissues (known as adventitious shoot formation) or through pre-existing meristematic tissues (known as axillary shoot formation). A successful plant regeneration protocol requires appropriate choice of explant, age of the explant, definite media formulations, specific growth regulators, genotype, source of carbohydrate, gelling agent and other physical factors including light regime, temperature and humidity (Sujatha and Mukta, 1996; Sujatha *et al.*, 2005; Deore and Johnson, 2008).

The interest in secondary metabolites and oil from *Jatropha curcas* has generated enormous pressure to supply seedlings that are homogenous and

productive for establishment of Jatropha plantations. Therefore, there is a need to mass propagate elite trees. Seeds and cuttings are two conventional propagation methods for this plant. Conventional propagation of *J. curcas* is beset with problems of poor seed viability, low germination, scanty and delayed rooting of seedlings and vegetative cuttings (Heller, 1996; Openshaw, 2000). The culturing of plant cells or organs can overcome problems of the flowering season, pollination, pollinators, seed setting, gestation period, viral infections etc. Therefore there is a need to develop tissue culture protocols that can be used for production of quality planting material.

I.5. SOMACLONAL VARIATION

Plants are subjected to phenotypic or genotypic variation concomitant with changes in the environment. In 1958 a novel, artificially produced, source of genetic variability was reported by Steward, as higher plant cells cultured *in vitro* showed genetic instability. Plants regenerated from calli often display qualitative and quantitative phenotypic alterations, cytological abnormalities, sequence changes, and gene activation and silencing. These cell culture induced changes, collectively called somaclonal variation, may be stable or unstable, reversible or irreversible, meiotically reset or transgenerationally transmitted (Karp, 1991; Phillips *et al.*, 1994; Kaeppler *et al.*, 2000). The first observation of somaclonal variation was reported by Braun in 1959. Subsequently, the variability existing in plant tissue and cell cultures received much attention and neologisms were proposed by Larkin and Scowcroft (1981).

Somaclonal variation has been reported at different levels in micropropagated plants. It can be morphological, cytological, cytochemical, biochemical, and molecular variations (Rani and Raina, 2000). Epigenetic changes (mainly in DNA methylation levels) were reported in response to water deficiency (Labra *et al.*, 2002), osmotic stress (Kovarik *et al.*, 1997), and presence of heavy metals (Aina *et al.*, 2004). Variation in chromosome numbers and structures, and

chromosome irregularities (such as breaks, acentric and centric fragments, ring chromosomes, deletions and inversions) are observed during *in vitro* differentiation and among regenerated somaclones (Mujib *et al.*, 2007; Hao *et al.*, 2002). Cryptic changes, such as point mutations, are also expected to occur and may affect the chloroplast or mitochondrial genomes.

Somaclonal variation is being used for the improvement of many forest trees (Gyvess *et al.*, 2007; Rathore *et al.*, 2007) especially by *in vitro* induced mutagenesis and genetic transformation through callus culture. Induction of genetic variation depends on the plant species, the genotype involved, the type of explant, the age of culture, the culture media and the culture conditions (Karp and Bright, 1985; Shuangxia *et al.*, 2008).

I.5.1. Causes of somaclonal variation

Cellular organization is a critical factor for plant growth, whereas *in vitro* growth is characterized by the loss of cellular control (Karp, 1994; Sivanesan, 2007). The disorganized state of callus phase is widely assumed to be responsible for the higher rate of resultant somaclonal variation (Vazquez, 2001). Conditions of culture *in vitro* can be extremely stressful for plant cells and may initiate highly mutagenic processes (Kaeppler and Phillips, 1993). Generation of callus cultures involves cell adaptation to the conditions of *in vitro* culturing and requires changes that are beyond the reaction norm of the genome. This results in destabilizing selection and an increase in genetic variation during early stages of the culture (Kunakh, 1999).

In recent years considerable amount of evidence has accumulated indicating that all classes of plant hormones can cause selective changes in the levels of specific mRNAs (Baulcombe *et al.*, 1980; Higgins *et al.*, 1982; Christoffersenr and Laties, 1982).

Transposable elements (TEs) are one of the causes of genetic rearrangements in *in vitro* cultures. Transpositional events, such as the activation

of transposable elements, putative silencing of genes and a high frequency of methylation pattern variation among single-copy sequences, play a role in somaclonal variation (Hirochika., 1993; Barret *et al.*, 2006). Tissue culture is reported to activate silent TEs, resulting in somaclonal variations (Hirochika *et al.*, 1996). The triggers of mutations in tissue culture remain controversial (Joyce *et al.*, 2003) and numerous factors have been suggested on this aspect, including plant growth regulators, lighting conditions (George, 1993), aseptic conditions, imbalances of media components, the relationship between high humidity and transpiration (Cassells and Roche, 1994; Cassells and Walsh, 1994), saline stress, oxidative stress and nutrient deficiency (Joyce *et al.*, 2003).

I.5.1.1. Explant source

The use of meristematic tissues, such as the pericycle, procambium and cambium, as starting materials for tissue culture reduces the possibility of variation (Sahijram *et al.*, 2003). In contrast, highly differentiated tissues, such as roots, leaves, and stems, generally produce more variants, probably due to the callus-phase, than explants that have pre-existing meristems (Sharma *et al.*, 2007).

I.5.1.2. Plant growth regulators

Unbalanced concentrations of auxins and cytokinins may induce polyploidy, whereas under a low concentration or total absence of growth regulators the cells show normal ploidy (Swartz, 1991). Auxins added to cultures of unorganised calli or cell suspensions increase genetic variation by increasing the DNA methylation rate (LoSchiavo et al., 1989). It would seem that growth regulators preferentially increase the rate of division of genetically abnormal cells (Bayliss, 1980). An increase in number of chromosomes in presence of high level benzyladenine (BA) has been observed in the banana cultivar 'Williams' by Gimenez et al. (2001).

I.5.1.3. Genotype

Rapid multiplication of a tissue or long-term cultures may affect genetic stability and thus lead to somaclonal variation (Israeli *et al.*, 1995). An interaction between genotype and the tissue culture environment is also reported by Martin *et al.* (2006). Different genomes respond differently to the stress-induced variation, which indicates that somaclonal variation also has genotypic components (Leva *et al.*, 2007). The differences in genetic stability are related to differences in genetic make-up, because some components of the plant genome may become unstable during the culture process. For example, the repetitive DNA sequences can differ in quality and quantity between plant species (Lee and Phillips, 1988).

OBJECTIVES OF THE PRESENT STUDY

Given the economic importance of *Jatropha curcas*, and its limited availability in the wild, it would be desirable to establish plantations of the tree so as to obtain assured supply of raw material for extraction of phytochemicals, and seeds for production of biodiesel. However both seed propagation as well as propagation by cuttings is unsatisfactory in this tree species. Seeds have poor viability and are genetically heterozygous leading to genetic variability in terms of growth, biomass, seed yield, and oil content. Stem cuttings have poor roots and the trees are easily uprooted. Tissue culture techniques could possibly be gainfully employed in the propagation of elite plants of *Jatropha*.

When plant tissue is passaged through *in vitro* culture, there is possibility of induction of variations. An estimation of somaclonal variability is useful in a determination of culture protocols. Molecular markers could be employed to estimate the amount of variations induced in callus and regenerants by different hormonal combinations used in culture.

In this context the present study aims to develop an *in vitro* propagation protocol for the production of plantlets and to evaluate the variation induced in callus and regenerants in comparison with mother plant by the use of molecular markers and by studying phytochemicals and bio active compounds present in callus and regenerated plants.

The specific objectives of the thesis therefore include the following:

1. To determine the effect of plant growth regulators on the induction of callus and regeneration of plants from leaf, nodal and petiole explants of *Jatropha curcas* L.

2. To determine the effect of plant growth regulators on cell cycle and mitotic index during callus induction from leaf explants.
3. To estimate the frequency of sequence variations induced in callus and regenerated plants using molecular markers.
4. To assess the variation in bioactive compounds in callus and regenerated plants.

CHAPTER II: STUDIES ON CALLUS INDUCTION AND PLANT REGENERATION IN JATROPHA CURCAS L.

II.1. REVIEW OF LITERATURE

II.1.1. *IN VITRO* PROPAGATION

In vitro techniques have been used for propagation of many plant species and to generate material for germplasm preservation, and crop improvement to increase field productivity and profitability (Thepsamran *et al.*, 2007). *In vitro* techniques are also required for studies on cell signalling, cytogenetics, genetic engineering and production of secondary metabolites. Tissue culture of *J. curcas* was undertaken to circumvent the problems associated with large scale multiplication (Sujatha and Mukta, 1996). *Jatropha* species are amenable to tissue culture manipulations, which indicate scope for widening the genetic base through parasexual hybridization and biotechnological tools (Sujatha, 2006). Evaluations of tissue culture propagated plants of *J. curcas* indicate that they have an advantage over seed propagated plants in terms of yield and yield related traits (Sujatha *et al.*, 2005). Farmers who adopt *in vitro* propagated material may benefit from increased income through reduced pest control costs and higher effective yields (Muyanga, 2009).

II.1.2. EFFECT OF GROWTH REGULATORS

Plant hormones play a crucial role in controlling the way in which plants grow and develop. They regulate the speed of growth of the individual parts and integrate these parts to produce the plants. Both auxins and cytokinins are synergistically required to induce cell division, differentiation and growth in plant tissue cultures (Shrivastava and Banerjee, 2008; Purkayastha *et al.*, 2010; Jha *et al.*, 2007). It is important to find out the triggering combination and concentrations of plant growth regulators besides other factors that vary from cell to cell even

within a particular type of tissue of a plant species. Ammirato (2004) reported that cytokinin at moderate concentrations enhance shoot development. Auxins have been shown to regulate different aspects of plant growth and development by affecting numerous processes, including cell division, cell elongation and differentiation (Davies, 1996)

Thidiazuron (TDZ), a urea-derived cytokinin, is a potent cytokinin for woody plant tissue culture (Huetteman and Preece, 1993) and is extensively used for the induction of shoot regeneration in several plant species (Li *et al.*, 2000; Mohan and Krishnamurthy, 2002; Liu *et al.*, 2003). TDZ emerged as an effective bioregulant in cell and tissue cultures in wide array of plant species (Li *et al.*, 2000; Hosseini-Nasr and Rashid, 2000; Svetla *et al.*, 2003; Matand and Prakash, 2007). Zhang *et al.* (2001), Ipekci and Gozukirmizi (2003), Panaia *et al.* (2004) and Belokurova *et al.* (2004) have reported that the application of TDZ, is an effective growth regulator for induction of somatic embryogenesis. The presence of TDZ in the induction medium has greater influence on the formation of adventitious shoot buds, whereas BAP in the absence of TDZ promoted callus induction rather than shoot buds (Deore and Johnson, 2008). The exogenous application of TDZ affects concentration of endogenous plant growth regulators in some members of dicots. TDZ affects pathways of purines and cytokinin metabolism (Mok *et al.*, 1982; Capelle *et al.*, 1983; Laloue and Fox, 1989).

II.1.3. DIRECT REGENERATION

Different combinations of cytokinins such as benzylaminopurine (BAP), kinetin (Kn) and auxins like naphthyl acetic acid (NAA), indolebutyric acid (IBA) as well as indole-3-acetic acid (IAA) control direct adventitious multiple shoot bud generation from epicotyl, hypocotyl, petiole and cotyledon of *J. curcas* (Sujatha and Mukta, 1996; Sujatha *et al.*, 2005; Cho *et al.*, 2007; Deore and Johnson, 2008).

Direct regeneration from hypocotyl, petiole and leaf explants of *Jatropha curcas* L. was reported by Sujatha and Mukta (1996) on various concentrations of zeatin, kinetin and N 6 benzyladenine (BAP) either singly or in combination with indole-3-butyric acid (IBA). Rate of regeneration from hypocotyl and petiole explants seemed to be higher in BAP with IBA combination than in zeatin or kinetin supplemented media. Leaf discs from the third expanding leaf exhibited higher regeneration potential than those from the fourth leaf. Rajore and Batra (2005) used shoot tip explant for regeneration of *Jatropha curcas*. Shoots were formed in MS medium with addition of BAP (2.0 mg/L) and IAA (0.5 mg/L) along with adenine sulphate, glutamine and activated charcoal. A suitable medium for *in vitro* multiple shoot induction of *Jatropha curcas* L. from axillary bud-derived shoots, about 0.7 cm., was reported by Thepsamran *et al.* (2007). In Murashige and Skoog (MS) medium incorporated with different concentrations of BAP alone and in combinations with IBA shoot proliferation was obtained. The combination 0.049 µM IBA and 2.22 µM BAP was reported by them to be the best medium for shoot multiplication

Nodal meristems are found to be an important source tissue of micropropagation and plants raised from these are comparatively more resistant to genetic variation (Pierik, 1991). Datta *et al.* (2007) achieved *in vitro* clonal propagation of seven month- old *J. curcas* employing nodal explants. Axillary shoot bud proliferation was best initiated on Murashige and Skoog's (MS) basal medium supplemented with 22.2 µM N6- benzyladenine (BAP) and 55.6 µM adenine sulphate, in which cultures produced 6.2 ± 0.56 shoots per nodal explant with 2.0 ± 0.18 cm average length, after 4–6 weeks. The rate of shoot multiplication was significantly enhanced after transfer to MS basal medium supplemented with 2.3 µM 6-furfuryl amino purine (Kinetin), 0.5 µM indole- 3-butyric acid (IBA) and 27.8 µM adenine sulphate for 4 weeks. Shoots proliferated on B5 medium supplemented with 1.0 mg/L benzylamino-purine, 1.0 mg/L

Kinetin and 2.0 mg/L naphthaleneacetic acid resulted the highest number of shoots (6.6 shoots per explant) (Warakagoda and Subasinghe, 2009)

In a study by Kalimuthu *et al.* (2007) nodal explants were used for micropropagation on MS supplemented with BAP (1.5 mg/L), Kn (0.5 mg/L) and IAA (0.1 mg/L). Kumar and Reddy (2010) employed direct shoot buds induction method from petiole explants, without the formation of an intervening callus using a Murashige and Skoog (MS) medium supplemented with different concentrations of thidiazuron (TDZ). The best induction of shoot buds (58.35%) and the number of shoot buds per explant (10.10) were observed when *in vitro* petiole explants were placed horizontally on MS medium supplemented with 2.27 µM TDZ after 6 weeks. Kumar *et al.* (2010) and Panghal *et al.* (2012) developed a protocol for the direct regeneration from cotyledonary leaf explants and petiole in TDZ supplemented medium. Tissue-culture protocols for endosperm cultures and the rapid propagation of selected genotypes of Jatropha have been reported by various researchers (Srivastava and Johri, 1974; Sujatha and Dhingra, 1993; Sujatha and Reddy, 2000). Adventitious shoot buds were induced from very young leaf explants of *in vitro* germinated seedlings as well as mature field-grown plants cultured on Murashige and Skoog's (MS) medium supplemented with thidiazuron (TDZ) (2.27 µM), 6-benzylaminopurine (BAP) (2.22 µM) and indole-3-butyric acid (IBA) (0.49 µM) (Deore and Johnson, 2008). A rapid and reproducible protocol for direct shoot regeneration from different explants of *Jatropha integerrima* was developed by Sujatha and Mukta (1993). In combination of 2.2 or 4.4 µM BAP and 4.9 µM IBA adventitious shoot bud initiation was obtained. In a study by Qin *et al.* (2004) epicotyl explants from *Jatropha curcas* were cultured and in combination, IBA 0.1 mg/L and BAP 0.2-0.7 mg/L, adventitious buds were obtained from the surface of epicotyl explants without callus formation. Highest regeneration frequency was reported in media IBA 0.1 mg/L and BAP 0.5 mg/L.

In a study by Sujatha and Sulekha (2007) sprouting of buds from axillary meristems of *Pongamia pinnata,* another biofuel tree was 64% on MS medium

devoid of plant growth regulators. Incorporation of BAP, Kn, or Zeatin was ineffective and sprouting was completely suppressed in the presence of TDZ. Caulogenic buds appeared in nodal meristems of these explants after withdrawal of TDZ.

II.1.4. INDIRECT REGENERATION

Globular, lush, soft and friable callus cultures were initiated from leaf and hypocotyl explants isolated from 4 days old seedling of *Jatropha curcas* L., on MS supplemented with 0.5 mg/L 2, 4-D with 2% v/v coconut milk by Soomro and Memon (2007). In their study, callus produced from hypocotyl explants grew faster and could establish homogeneous and chlorophyllous suspension culture from this calli in basal medium supplemented with 2,4-D, BAP, GA_3 and coconut milk.

Liu *et al.* (2003) developed a protocol for callus induction from petiole, hypocotyls and leaf blade followed by shooting. Sujatha *et al.* (2005) developed a method for the differentiation of adventitious shoot buds interspersed with callus from vegetative explants of nontoxic *J. curcas*. It is also reported that regeneration in *J. curcas* is highly genotype dependent (Machado *et al.*, 1997). Weida *et al.* (2003) reported shoot bud induction through callus on Murashige and Skoog's (MS) medium supplemented with 0.5 mg/L 6-benzylaminopurine (BAP) and 1.0 mg/L indole-3-butyric acid (IBA). Callus mediated shoot regeneration was obtained from the combinations of IBA 0.5 mg/L with BAP 0.1 mg/L, IBA 0.5 mg/L with BAP 0.2 mg/L and IBA 1.0 mg/L with BAP 0.5 mg/L, and the most appropriate combination was found to be IBA 1.0 mg/L and BAP 0.5 mg/L (Qin *et al.*, 2004). 2.22 µM BAP with 0.49 µM IBA concentration proved effective in callus mediated regeneration from hypocotyl and leaf explants, whereas lower concentration, was required for the petioles (0.44 µM BAP and 0.49 µM IBA) (Sujatha and Mukta, 1996). Fayyaz *et al.* (1994) also developed callus induction protocol from hypocotyls explants in 2,4-D and coconut milk containing medium.

Tiwari *et al.* (2002) reported that callus establishment through nodal explants and shoot proliferation through callus in *J. curcas* is difficult due to problems related to endogenous contamination and presence of phenolic compounds in explants. They induced massive and profuse green, fragile callus in MS culture medium supplemented with BAP 4.0 mg/L with 2, 4-D 1.0 mg/L within 3 to 4 weeks of culture period. When the callus was treated with BAP 5.0 mg/L and IAA 3.0 mg/L shoot proliferation could be attained. Number of shoots induced increased (7 to 8 shoots) on subculturing in medium of the same composition with a periodicity of four weeks.

Varshney and Johnson (2010) used immature embryos (1.1–1.5 cm) obtained from the *J. curcas* fruits, 6 weeks after pollination. On MS medium supplemented with IBA (0.5 mg/L) and BAP (1.0 mg/L) they showed a good response of morphogenic callus induction (85.7%) and subsequent plant regeneration (70%) with the maximum number of plantlets (4.7/explant). In the presence of growth adjuvants such as 100 mg/L casein hydrolysate 200 mg/L L-glutamine and 8.0 mg/L $CuSO_4$ frequency of callus induction increased (100%).

II.1.5. SOMATIC EMBYOGENESIS

Jha *et al.* (2007) were the first to successfully apply somatic embryogenesis to regenerate plants in *J. curcas*. Embryogenic calli were obtained from leaf explants on MS basal medium supplemented with 9.3 μM Kinetin (Kn). Induction of globular somatic embryos from 58% of the cultures was achieved on MS medium with different concentrations of 2.3–4.6 μM Kn and 0.5–4.9 μM IBA. A combination of 2.3 μM Kn and 1.0 μM IBA proved to be the most effective for somatic embryo induction in *J. curcas*. Addition of 13.6 μM adenine sulphate stimulated the process of development of somatic embryos. Mature somatic embryos were converted to plantlets on half strength MS basal medium with 90% survival rate in the field condition. Li *et al.* (2010) established an experimental system of *in vitro* maturation and germination of *J. curcas* microspore. Their

results suggested that the most appropriate medium for *in vitro* culture of *J. curcas* microspores was MS salts with White's vitamins and 22% maltose and the maturation and germination rate were 39.5% and 2.7%, respectively.

II.1.6. ROOTING

In the study by Rajore and Batra (2005) highest rooting response was observed when *in vitro* produced shoots of *J. curcas* were transferred onto half strength MS medium with 3.0 mg/L IBA. The induction of rooting with IBA pretreatment in *J. curcas* supports the earlier findings in poplar (Gunes, 2000). Results of Kochhar *et al.* (2005) suggest that early metabolization of auxin rather than total auxin content affects the process of root initiation. In his study, two Jatropha species, *J. glandulifera* and *J. curcas* exhibited differential response to external auxin application, suggesting that endogenous auxin status of the cuttings makes selective response towards different auxins.

Regenerated shoots of *J. curcas* were rooted on MS medium with 2.46 µM IBA after 5 weeks, and then transferred to MS medium without plant growth regulator (Thepsamran *et al.*, 2007). About 52% of root induction occurred in MS basal medium supplemented with 1.0 µM IBA in 2–3 weeks. Further elongation of roots with average length of 8.7 ± 1.35 cm was obtained in unsupplemented MS basal medium for 2–3 weeks. The plantlets (12–16 week old) were successfully acclimatized in soil with 87% survival frequency (Datta *et al.*, 2007). In a study by Sugla *et al.* (2007) shoots formed *in vitro* in *Jatropha curcas* were rooted on full-strength MS medium supplemented with 1.0 µM indole butyric acid (IBA).

Regenerated shoots of *Jatropha integerrima,* rooted readily on MS medium lacking growth regulators (Sujatha and Mukta, 1993 and 1996, Qin *et al.*, 2004). Regenerated plants with well developed shoots and roots were successfully transferred to greenhouse, without visible detectable variation (Qin *et al.*, 2004). Sujatha and Sulekha (2007) reported that the plant growth regulator free medium worked for rooting in *Pongamia pinnata* also.

Nanda and Kochhar (1985) reported that a balance of auxin and carbohydrates determines the ability of cuttings to root. Direct rooting is reported in plants (Bhatia *et al.*, 2002; Martin *et al.*, 2003; Rajeswari and Paliwal, 2008). Tiwari *et al.* (2002) also could establish 62 percent survival of the plant by *ex vitro* rooting when green and healthy shoots with 3 to 4 nodes were harvested and shifted to poly huts under high humid condition.

The number of leaves is higher and the flowering time earlier when cuttings were treated with IBA (Kochhar *et al.*, 2008). A more interesting observation is that shoots are formed much earlier in Jatropha species than roots. Shoots thus formed earlier, due to reserve carbohydrates, start producing auxins which moves downward, thereby accumulating in the lower portion of the cuttings. When the concentration reaches a threshold value, endogenous auxins at the extreme basal end start getting metabolized and signals the process of root initiation (Kocchar *et al.*, 2005).

II.1.7. MITOTIC INDEX

The mitotic index (MI) defined as percentage of cells at mitotic phase relative to the total cells, is a positive reflection of the capacity of a cell population to grow: the greater the mitotic index, the faster a population grows (Wareing and Philips, 1981). Cell division, biomass production, besides others, is affected by growth regulators and composition of culture media (Forsyth and Staden 1986). Mitotic index studies are important in the process of freeze preservation, in order to detect its viability in short term. Cell proliferation rates in plant cell cultures affect the chemical profile and secondary metabolite production, consequently identification of factors affecting cell proliferation is important (Biondi and Thorpe, 1982). Long-term cultivated callus cultures are generally characterized by a low mitotic activity (Hlinkova and Ruzickova, 2000). During the early development in callus cultures and in actively dividing suspension cultures mitotic

index can provide evidence of division synchrony (Yeoman *et al.*, 1966; Yeoman and Evan, 1967; Street, 1968).

Hao *et al.* (2002) reported that the cell size can be used as morphological markers to calculate mitotic index and ploidy level of citrus callus. Studies by Apte *et al.* (1987) on the effects of ageing on electrokinetic potential and growth index showed a relationship between these two parameters and reported that the rate of change of electrokinetic potential with age could be employed as a parameter to study the growth kinetics of cells in callus cultures. The semi thin sections of callus of *Fagonia arabica* leaf explants were examined under light microscope and the mitotic indices (MI) and mitotic stage indices (MSI) were determined (Eman and Alam, 2010). Mitotic index studies of callus from *Paconia saffruticosa* was done in order to test the effect of subculturing and physical conditions of the medium on mitotic cycle kinetics of a population of cells (Demoise and Partanen, 1969).

II.2.1. PLANT MATERIALS

An elite clone of *Jatropha curcas* L. was collected from Enhanced Biofuels & Technologies India Private Limited, Coimbatore and grown in 12 inch pots in the Department of Biotechnology, Cochin University of Science and Technology (Figure 2.1).

Figure 2.1: *Jatropha curcas*, elite plant used for the current study

(TBGT 70000)

A specimen of the same was deposited in the herbarium of Jawaharlal Nehru Tropical Botanical Garden Research Institute (JNTBGRI), Trivandrum (Herbarium specimen number: TBGT- 70000) (Figure 2.2). The plants were healthy and free of symptoms of disease, pest problems, showed good biomass yield and were used as the source of explants. In the present study plants were generated from *in vitro* cultures either "directly" from the explants or "indirectly" from callus induced from the explants.

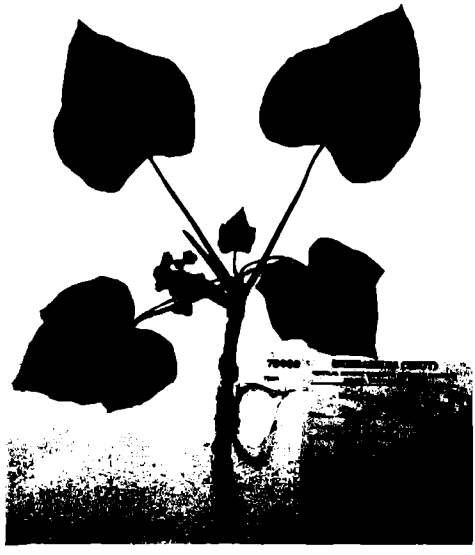


Figure 2.2: Specimen of *Jatropha curcas* L. (TBGT 70000) deposited in the herbarium of Jawaharlal Nehru Tropical Botanical Garden Research Institute (JNTBGRI), Trivandrum,

II.2.2. DIRECT REGENERATION

The nodal explants containing lateral buds were collected from 4-16 month old plants were used for the study direct regeneration.

II.2.2.1. Preparation of the explant

The explants were washed and kept under running tap water with a drop of tween 20 prior to surface sterilization. They were surface sterilized in 0.1% Mercuric chloride (w/v) for 15 minutes followed by repeated washing (3 times) with sterile distilled water. After sterilization they were trimmed (1- 1.5 cm), and inoculated in the culture medium.

II.2.2.2. Culture media

Murashige and Skoog (MS) (1962) (Appendix 1) basal medium was used for the culturing. The medium was supplemented with 3% (w/v) sucrose (Analytical grade, Himedia, India). Stock solutions of sulphates, non-sulphates and vitamins were prepared by dissolving required quantity of chemicals in distilled water. Growth regulators were prepared by adding 2-3 drops of 1N NaOH / 1N HCl to 100 mg of auxin/ cytokinin until solubilisation of the crystals. The volume was made up to 100 ml with sterile distilled water. The stock solutions were stored at 4^0C.

For the induction of adventitious shoots from nodal explants, combinations of Gibberellic acid (GA_3) and 6-Benzylaminopurine (BAP) were used (Table 2.1). All media were solidified with 0.8% (w/v) agar (Hi media, India). The pH of media was adjusted to 5.7±0.1 prior to autoclaving at 121^0C at 1.1kg sq.cm for 15 minutes. Cultures were incubated under 16h/8h light/dark cycles at 23±2^0C. Shoots formed from nodal explants were sub cultured onto same medium. The frequency of shooting response was recorded as percent of the shoots formed per explant.

$$\text{Percentage of shooting response} = \frac{\text{No: of shoots induced}}{\text{Total no. of explants inoculated}} \times 100$$

Table 2.1: **Phytohormones used for induction of shoots from nodal explants of *J. curcas***

S. No.	Hormonal combinations BAP+GA_3 (mg/L)
1	1.0 + 0.2
2	0.8+ 0.2
3	0.8+0.4
4	1.0+0.4
5	0.8+0.6

II. 2.3. CALLUS MEDIATED INDIRECT REGENERATION

Leaf and petiole were used for callus mediated indirect plant regeneration of *J. curcas* L. The explants were collected from 4 to 16 month old plants. The 2nd and 3rd leaves from the apex served as source of leaf discs and petioles.

II.2.3.1. Preparation of the explant

Explants were washed well and kept under running tap water with a drop of tween 20 prior to surface sterilization in 0.1% Mercuric chloride (w/v) for 15 minutes. They were washed thoroughly with sterile distilled water for three times to remove traces of surface sterilant. The petioles were cut into segments of ~3mm and inoculated. The leaf explants were trimmed into discs of ~3mm in diameter and placed with the abaxial side in contact with the medium.

II.2.3.2 Culture media

The Murashige and Skoog (MS), 1962 (Appendix 1) basal medium supplemented with 3% (w/v) sucrose (Analytical grade, Himedia, India) augmented with different hormonal combination was used for the induction of callus.

II.2.3.3. Induction of callus

Callus was induced on MS basal medium fortified with different combinations of auxins [Indole butyric acid (IBA) and Indole acetic acid (IAA)], cytokinin [Kinetin (Kn) and Benzylaminopurine (BAP)] and Adenine sulphate (AS) as detailed in Table: 2.2. 0.8% (w/v) agar (Bacteriological grade, Himedia, India) used as gelling agent for the media. The pH of media was adjusted to 5.7±0.1 prior to autoclaving at 121^{0}C at 1.1kg sq.cm for 15 minutes. The experiments were carried out in culture tubes (150×25 mm) containing 20 ml of culture medium. Cultures were maintained in the culture room under fluorescent light of 16h photoperiod and 8 h dark periods at a temperature at 23±2^{0}C. The frequency of response was recorded as percent of the formation of callus from explants.

$$\text{Percentage of callus response} = \frac{\text{No: of callus induced}}{\text{Total no. of explants inoculated}} \times 100$$

The callus was sub cultured into the same media at intervals of 30 days.

Table 2.2: Phytohormones used for induction of callus from leaf discs and petiole of *J. curcas*

S. No.	Hormones	Concentration mg/L
1	Kn	1.0
2	Kn	2.0
3	Kn+IAA+BA	0.5+0.3+1.0
4	IBA+BAP+AS	0.2+4.0+20.0
5	BAP+AS	5.0+20.0
6	IBA+BAP	0.0+2.0
7	IBA+BAP	0.0+3.0
8	IBA+BAP	0.2+2.0
9	IBA+BAP	0.3 +2.0
10	IBA+BAP	0.3 +.5.0
11	IBA+BAP	0.4+1.0
12	IBA+BAP	0.4+1.5
13	IBA+BAP	0.4+2.0
14	IBA+BAP	0.4+2.5
15	IBA+BAP	0.4 +3.0
16	IBA+BAP	0.4+4.0
17	IBA+BAP	0.5+5.0
18	IBA+BAP	0.6+2.0

II.2.3.4. Callus mediated shoot induction and multiplication from leaf explants

The leaf discs were cultured in MS basal medium, consist of 3% w/v sucrose and 0.8% w/v agar, supplemented with different combinations of auxin (IBA) and cytokinin (BAP). The concentration of auxins varied from 0- 0.6 mg/L and of cytokinins from 1mg/L- 4 mg/L (Table: 2.3). The media was solidified with 0.8% (w/v) agar (Hi media, India). Shoots formed from the callus were sub cultured onto same medium. The experiments were carried out in culture tubes (150×25 mm) containing 20 ml of culture medium. Each combinational experiment was done in triplicate of 60 culture samples. Cultures were incubated in constant temperature growth room (23±2°C) fitted with lights to provide 16h/8h light/dark cycles. The frequency of shooting response was recorded as percent of the shoots formed per explant.

$$\text{Percentage of shooting response} = \frac{\text{No: of shoots induced}}{\text{Total no. of explants inoculated}} \times 100$$

Table 2.3: Phytohormones used for callus mediated shoot induction from leaf discs of *J. curcas*

S. No.	IBA+ BAP Conc.(mg/L) in MS media
1	0.0 +2.0
2	0.2 +2.0
3	0.3 +1.0
4	0.3+.2.5
5	0.4+2.0
6	0.4+2.5
7	0.4 +4.0
8	0.6+2.0

II.2.3.5. Microscopical examination of callus and organogenesis

Sections of the callus formed in MS supplemented with IBA and BAP were stained with Safranin and Giemsa in order to observe the stages of development under microscope.

II.2.4. INDUCTION OF NODULAR CALLUS USING THIDIAZURON

In an attempt to regenerate plants through somatic embryogenesis, leaf discs were cultured in MS basal medium fortified with different combinations of auxin [Indole butyric acid (IBA)] and Thidiazuron (TDZ) as cytokinin as detailed in Table 2.4. The medium was solidified with 0.8% (w/v) and agar (Himedia, India) and the pH adjusted to 5.7 ± 0.1 prior to autoclaving at 121^{0}C at 1.1kg sq.cm for 15 minutes.

II.2.4.1. Microscopical examination of the nodular structures

Sections of the nodular shoot buds formed in Thidiazuron containing media were stained with safranin in order to observe the different stages of development under microscope.

Table 2.4: Phytohormones used for induction of nodular callus from leaf discs of *J. curcas*

S. No.	Hormonal concentration-TDZ+IBA (mg/L)
1	1.0+0
2	1.0+0.3
3	1.0+0.4
4	1.5+0.3
5	2.0+0
6	2.0+0.2
7	2.0+0.3
8	2.0+0.4
9	2.0+0.5
10	2.0+0.6
11	2.5+0.3
12	2.5+0.4
13	3.0+0
14	3.0+0.4
15	3.0+0.5
16	3.0+0.6
17	3.5+0.4
18	4.0+0.4

II.2.5. ROOT INDUCTION

The *in vitro* developed single/multiple shoots (1.5 - 3.0 cm long) were excised and implanted in culture tubes with different treatments as given in Table: 2.5 under aseptic conditions for *in vitro* rooting.

Table 2.5: Treatments used for induction of roots in *in vitro* regenerated shoots of *J. curcas*

S.No.	Treatments
1	MS + IBA 0.2+ BAP 1.0 mg/L
2	MS +IBA-0.2mg/L
3	½ strength MS media
4	½ strength MS media + 0.2+0.0 (IBA+ BAP) mg/L
5	¼ strength MS media 0.3+0.0 (IBA+ BAP) mg/L
6	Direct shoot transfer in sterile Neopeat
7	Water agar medium (0.8%)
8	MS liquid media
9	MS basal with charcoal

II.2.6. ACCLIMATIZATION

After development of sufficient roots, the plantlets were gradually pulled out from the medium using forceps with extreme care to avoid any mechanical damage to the plantlets. They were washed thoroughly with sterile distilled water to remove all the traces of the media and treated with 0.5% Bavistin for 15 minutes to protect them from fungal attack. Then they were transplanted to small plastic cups containing sand, soil and neopeat (2:1:1 ratio) and kept in mist house under humidity range of 70-90% for two weeks for subjecting them to hardening. After that the plantlets were transferred to small pots containing sand and soil (2:1 ratio) and kept under humidity range of 60-70% in shade house.

II. 2. 7. STATISTICAL ANALYSIS

The experiments were carried out in culture tubes (150×25 mm) containing 20 ml of culture medium. Each combinational experiment was done in triplicate of 20 culture samples, in case of nodal culture and 60 for leaf disc cultures. The experiments were set up in a completely randomized design (CRD). Data were analyzed using SPSS (Statistical package of social science) Version: 17. The significant difference between means was detected using *ANOVA*. Means differing significantly were compared with 95% confidence interval at 5% probability level ($p \leq 0.5$) using *Posthoc test*. Variability of data was expressed as mean± standard error (SE)

II.2.8. MITOTIC INDEX IN CALLUS CULTURES

In order to elucidate the effect of phytohormones on the induction of cell division and proliferation of callus from leaf disc explants, the mitotic index was studied.

II.2.8.1. Specimen preparation

Callus induced on MS media with different hormonal concentrations was collected at 5 min time interval from 8.00 AM to 11.00 AM on the 2nd and 3rd day of subculturing and washed with distilled water. Pre- treatment was done with different concentrations of Colchicine (0.2-0.6%) for 3 to 24 h, at 4°C and room temperature in order to optimize the metaphase arrest. The callus was washed twice with distilled water to remove the traces of colchicine and then treated with hypotonic solution 0.075 M Potassium chloride for 30 min at 25°C. Fixation was done in a mixture of 3 parts of methanol and 1 part of glacial acetic acid for 4 h at 4°C. After washing twice with distilled water, the callus was macerated in 5 N HCl at 60°C for 5 min and again washed with distilled water.

II.2.8.2. Staining

The material was taken on a clean glass slide and crushed in a drop of 2% acetocarmine with the flat end of a rod and squashed under a coverslip by applying pressure under several thickness of blotting paper. Sideways movement of coverslip was avoided in order to prevent the rolling of cells. Finally the slide was mounted and examined under 10X, 40X and 100X. For scoring of different phases of chromosomes, temporary slides were used. Photographs were taken under 40X and oil immersion lens.

II.2.8.3. Scoring of slides

The mitotic index (MI) was determined as a ratio between the number of cells in mitosis and the total number of analysed cells. The index of each phase of mitotic division was calculated. The results were expressed as the mean of 3 replicates ± standard deviation (SD).

II.3.1. DIRECT REGENERATION OF *J. CURCAS* FROM NODAL EXPLANT

After 2 weeks of initial culture, nodal explants containing lateral buds cultured on MS basal medium supplemented with 1.0 mg/L BAP and 0.4 mg/L GA_3 developed multiple shoots (Figure 2.7) at a frequency of 60.0 % (Figure 2.3) with 3.6 shoots of height 10.0 mm, with number of leaves 6.0 (Figure 2.6). In the combination of 0.8 mg/L BAP and 0.2 mg/L GA3, explants gave elongated shoots of 11.0 mm (Figure 2.4). In the combinations used the number of shoots varied from 2.0 (1.0 mg/L BAP and 0.2 mg/L GA_3) to 4.0 (0.8 mg/L BAP and 0.2 mg/L GA_3) (Figure 2.5). Percentage of response was low in 0.8 mg/L BAP and 0.4 mg/L GA_3 (2.33)

II.3.1.1. Statistical analysis

Statistical analysis of variance showed that, the percentage of response is significantly high ($p<0.001$) in 1.0 mg/L BAP and 0.4 mg/L GA_3, when compared to 1.0 mg/L BAP and 0.2 mg/L GA_3, 0.8 mg/L BAP and 0.2 mg/L GA_3 and 0.8 mg/L BAP and 0.4 mg/L GA_3. There is only less significant variation ($p<0.05$) in percentage of response in the medium 1.0 mg/L BAP and 0.4 mg/L GA_3 (60.0%) and 0.8 mg/L BAP and 0.4 mg/L GA_3 (51.0%). And there is no significant variation in between 1.0 mg/L BAP, 0.8 mg/L GA_3 (38.3%) and 0.8 mg/L BAP, 0.2 mg/L GA_3 (40.0%) treatments.

There is no significant variation in the height of the shoot and number of shoots between the treatments ($p>0.05$). Combinations 0.8 mg/L BAP, 0.2 mg/L GA_3 and 1.0 mg/L BAP and 0.4 mg/L GA_3 with number of leaves 6.6 and 6.0 respectively showed a slight significant variation ($p<0.05$) from 0.8 mg/L BAP, 0.4 mg/L GA_3 (4.0) and 0.8 mg/L BAP and 0.6 mg/L GA_3 (3.6).

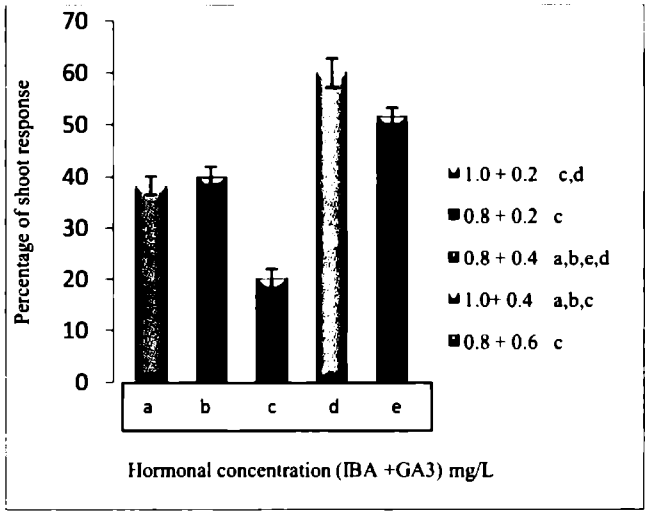


Figure 2.3: Percentage of shoot response from nodal explants in different hormonal combinations. Values represent the means ± SE. Letters to the right of the legend indicate significant differences varying ($p<0.05$) based on Tukey's *Post hoc* test.

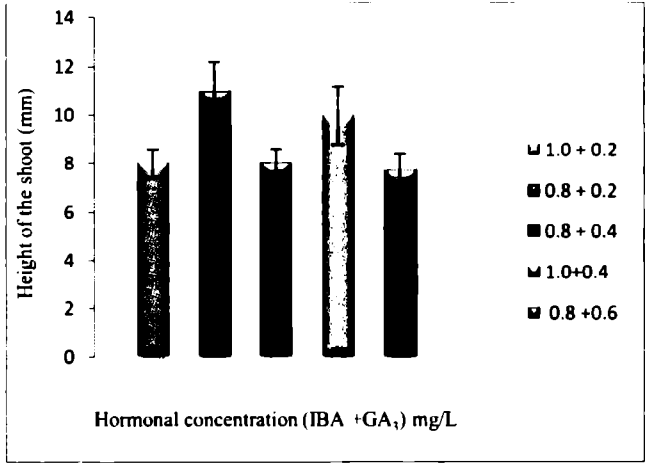


Figure 2.4: Effect of hormonal concentrations on length of the shoot. Values represent the means ± SE. No significant difference ($p<0.05$) based on Tukey's *Post hoc* test.

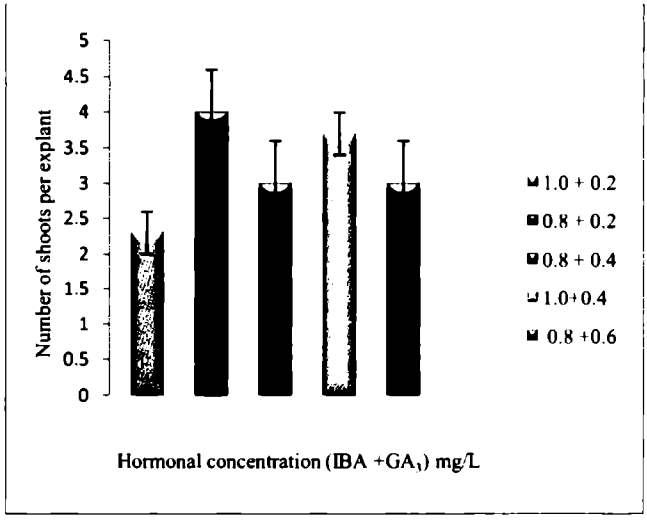


Figure 2.5: Effect of hormonal concentrations on number of shoot formation. Values represent the means ± SE. No significant difference ($p<0.05$) based on Tukey's *Post hoc* test.

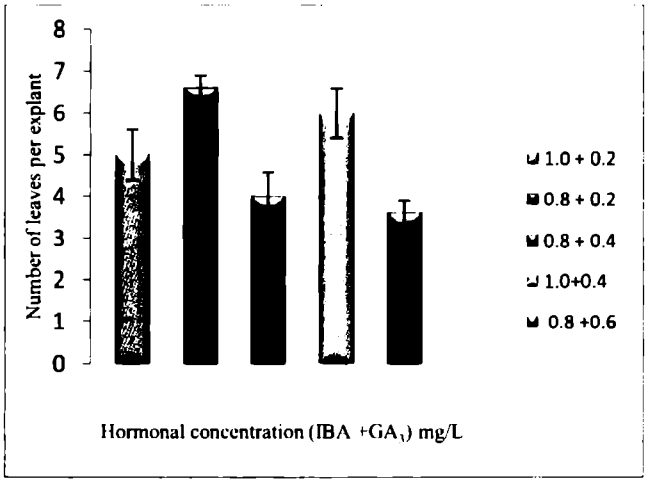


Figure 2.6: Effect of hormonal concentrations on number of leaves per shoots. Values represent the means ± SE. No significant difference ($p<0.05$) based on Tukey's *Post hoc* test.

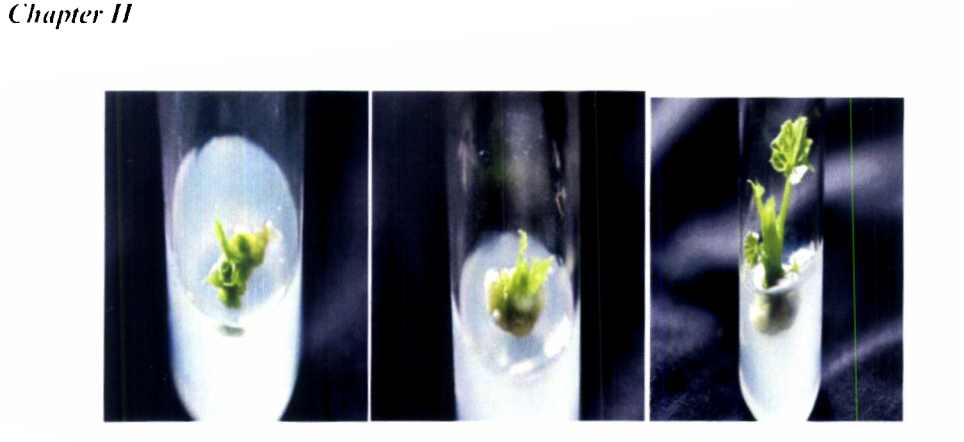


Figure 2.7: Direct regeneration of *J. curcas* nodal explants in MS with 1.0 mg/L BAP and 0.4 mg/L GA_3

II. 3.2. CALLUS INDUCTION

Response of the culture varied depending on the explant and hormonal combinations used. Variation in response, duration taken for the initial response, morphological characteristics, and rate of growth were observed. Leaf explant yielded better callus than petiole explants. Shoots could be regenerated from the callus.

II.3.2.1. Callus induction from petiole

The highest percentage (60.0%) of callus induction from petiole explants was given by 0.6 mg/L IBA and 2.0 mg/L BAP, whereas it was 91.6% in the same combination for the leaf disc (Figure 2.8). The combination showed significant variation ($p<0.05$) in percentage of response with all other combinations except 2.0 mg/L Kinetin and 0.2 mg/L IBA and 2 mg/L BAP (Appendix 2.a). 3.0 mg/L Kinetin gave white friable callus with 54.0% response. Callus formed from the petiole was soft and friable (Figure 2.9), white to light green in colour. Petiole showed slow rate of growth in comparison with leaf discs. The cell differentiation was visible only from the 8^{th} day of inoculation. Results indicate that Adenine sulphate does not have much effect on callus induction.

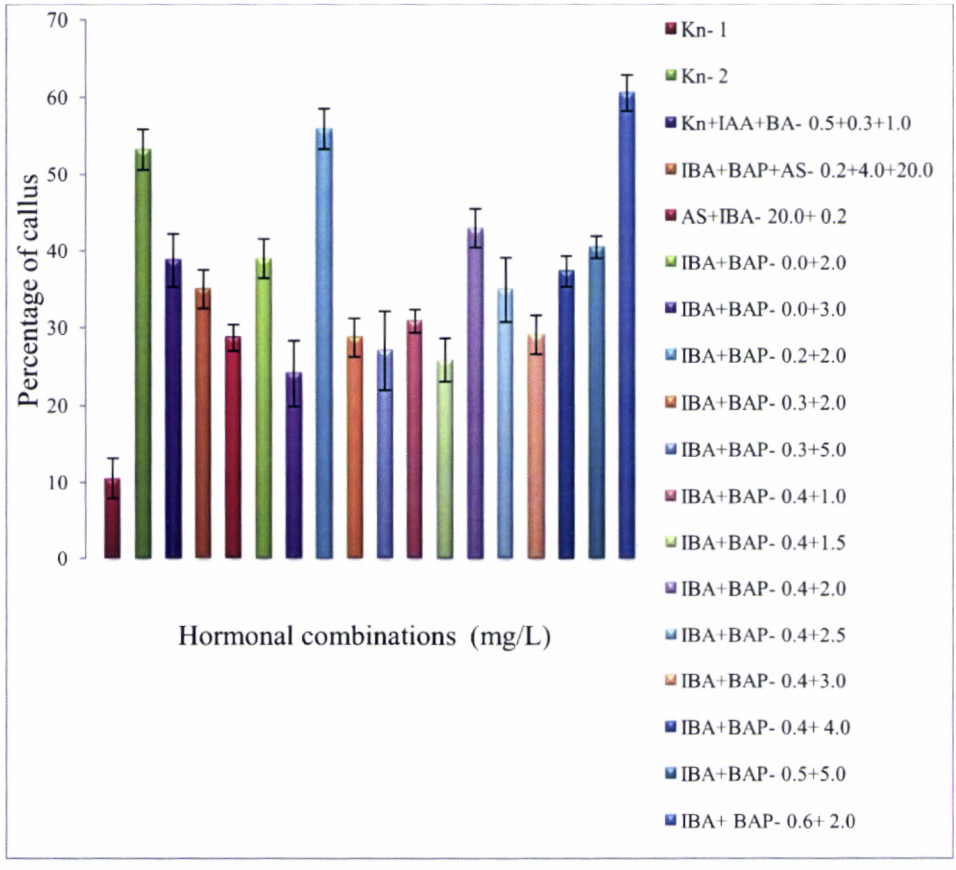


Figure 2.8: Percentage of callus response from petiole explants in different hormonal combinations.

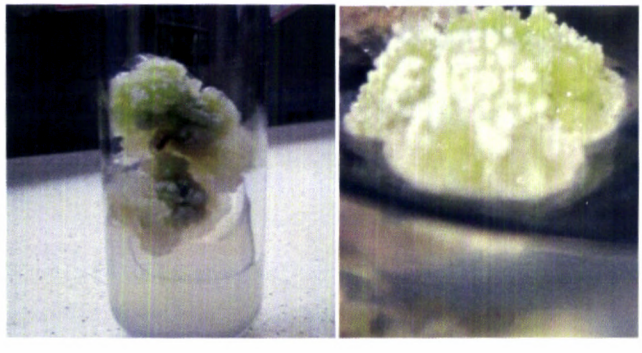


Figure 2.9: Callogenesis from petiole on 0.4 mg/L IBA and 1.5 mg/L BAP and 0.6 mg/L IBA and 2.0 mg/L BAP

II.3.2.2. Callus induction from leaf explants

In various combinations of growth hormones tested as detailed in Table 2.2, the explants showed profuse callusing. Leaf discs placed on the induction medium enlarged and exhibited varied response. The cell differentiation was visible from the 3rd day of inoculation. Explants started callusing within 2 weeks. The callus formed was different in various concentrations of growth hormones and ranged from dark green to light green and white and from compact to friable (Figure 2.11). In some cases, the explants showed only leaf expansion.

Best callus response was observed in MS media with 1.5 mg/L BAP and 0.4 mg/L IBA (Figure 2.10). The combination showed significant variation ($p<0.05$) in percentage of response with all other combinations except 0.3 mg/L IBA and 5.0 mg/L BAP, 0.4 mg/L IBA and 3.0 mg/L BAP, 0.4 mg/L IBA and 4.0 mg/L BAP and 0.6 mg/L IBA and 2.0 mg/L BAP (Appendix 2.b). Among other hormonal combinations tested, good callus response was observed in combinations 0.4 mg/L IBA and 1.0 mg/L (89.7%), 2.5 mg/L (91.1%), 3.0 mg/L (82.2%), and 4.0 mg/L BAP (91.07%). The experiments with the combination of adenine sulphate gave only expansion of leaf discs, and there was no callus formation. The callus formation was not evident in the absence of auxin as in the media containing 2.0 mg/L BAP. However shoot buds initiated in the same. The combination of 0.3 mg/L IAA and 0.3 mg/L BAP gave no response, while 0.3 mg/L IBA and 2.5 mg/L BAP gave 76.0% response.

As the concentration of IBA increases the compactness of callus increased. Combinations of IBA and BAP gave dark compact callus and showed adventitious shoot and bud induction within 45 days of leaf disc inoculation. The primary culture of callus on subculturing to same medium gave soft and friable callus which could be used to initiate suspension cultures.

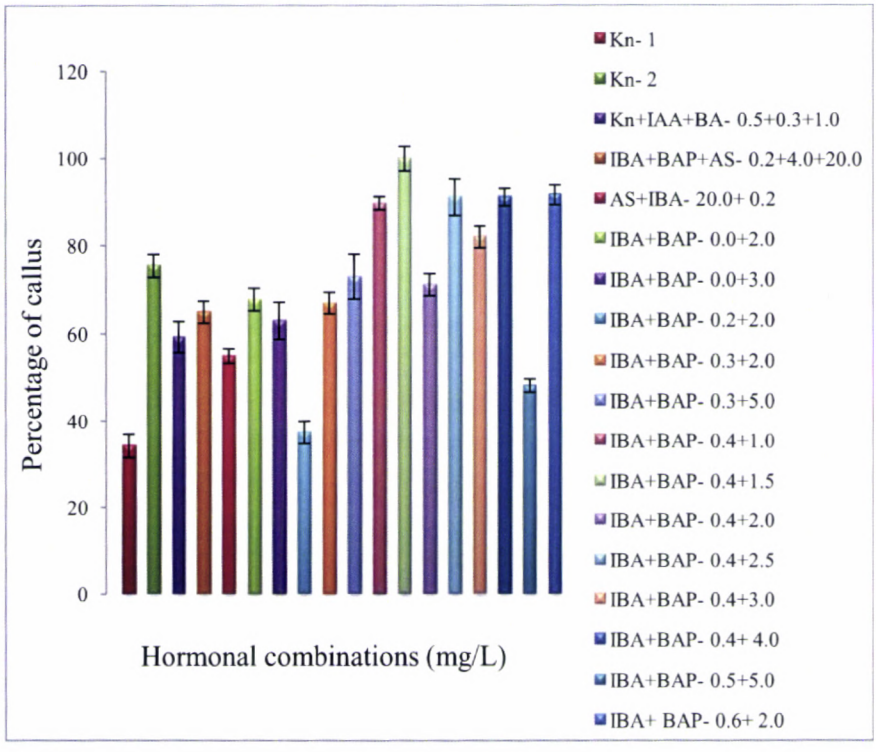


Figure 2.10: Percentage of callus response from leaf explants in different hormonal combinations.

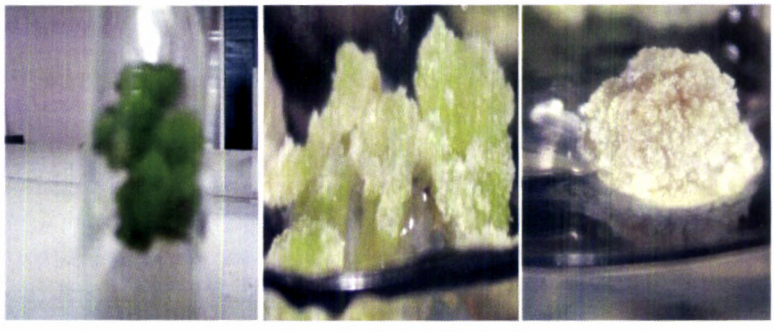


Figure 2.11: Callus of different morphological characteristics induced from leaf discs of *J. curcas* in various hormonal treatments

II.3.2.3. Microscopic examination of callus and organogenesis

The callus was compact and thus had to be teased with needles to study its cell types. The calli were heterogeneous in nature with wide variations in size and shapes of cells such as spheroidal, ovoid and elongated. Histogenetic differentiation in the form of tracheids was observed in calli formed from leaf explants, in some IBA, BAP containing MS media. Tracheids occurred singly or in groups and possessed scalariform thickenings on their walls (Figure 2.12)

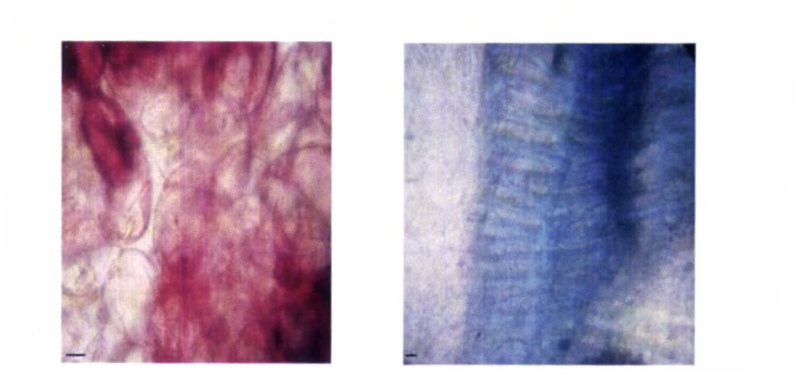


Figure 2.12: Photomicrographs showing callus cells and xylogenesis under 40 X (Bar represents 10μm)

II.3.3. INDIRECT REGENERATION FROM LEAF EXPLANT OF *J. CURCAS.*

The frequency of shoots and leaves formed from the leaf discs varied with the presence of both the cytokinin and auxin. Multiple shoot induction occurred within 4-6 weeks in the primary culture media (Figure 2.13). The best response (73.83%), seen in MS basal media with 0.4 mg/L IBA and 2.0 mg/L BAP combination (Figure: 2.14), which gave multiple shoots (3.16), of significantly higher ($p<0.05$) shoot height (14.20) when compared to 0.6 mg/L IBA and 2.0 mg/L BAP. 0.4 mg/L IBA and 4.0 mg/L BAP and 0. 2 mg/L IBA and 2.0 mg/L BAP also gave good responses with 13.77mm and 11.18 mm shoot lengths (Figure

2.15). The highest shoots per explant (~≥2mm) ratio, 3.73 with 66.1% of response were observed in the combination of 0.4 mg/L IBA and 4.0 mg/L BAP. The number of shoot per explant was significantly higher ($p<0.05$) in this combination when compared to 2.0 mg/L BAP and combination 0.2 mg/L IBA and 2.0 mg/L BAP. The combinations 0.6 mg/L IBA and 2.0 mg/L BAP and 0.3 mg/L IBA and 1.0 mg/L BAP also gave good response with average number of shoots per explant (~≥2 mm) 2.70 and 2.43 respectively (Figure 2.16). The best synergistic effect on number of leaves (8.34) seen in 0.4 mg/L IBA and 4.0 mg/L BAP combination, in which the number of leaves was significantly higher ($p<0.05$) than the treatments 2.0 mg/L BAP and combination 0.6 mg/L IBA and 2.0 mg/L BAP. It was found to be more also in MS supplemented with 0.4 mg/L IBA and 2.0 mg/L BAP (7.51) and 0.2 mg/L IBA and 2.0 mg/L BAP (6.31) (Figure 2.17).

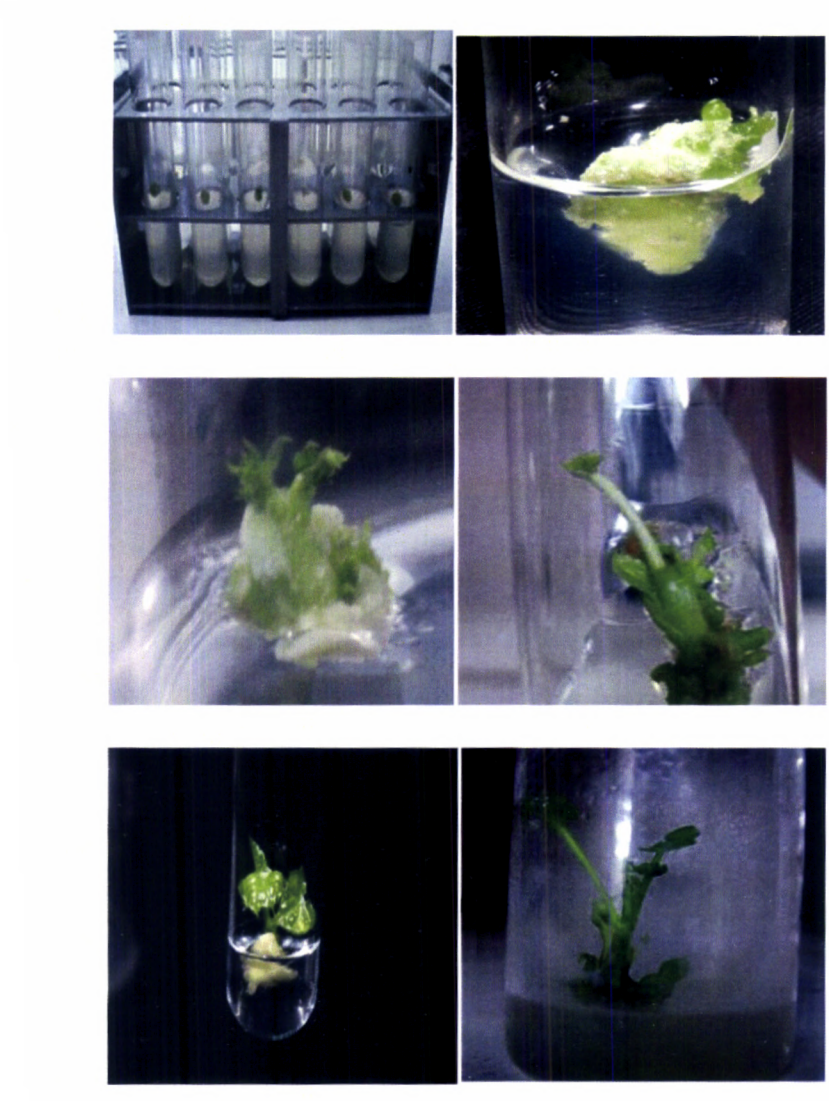


Figure 2.13: (a) Leaf disc explants (b) Callus showing shoot bud induction (c) Shoot initiation (d) Regenerated shoots (e) Shoot multiplication (f) Elongated shoots.

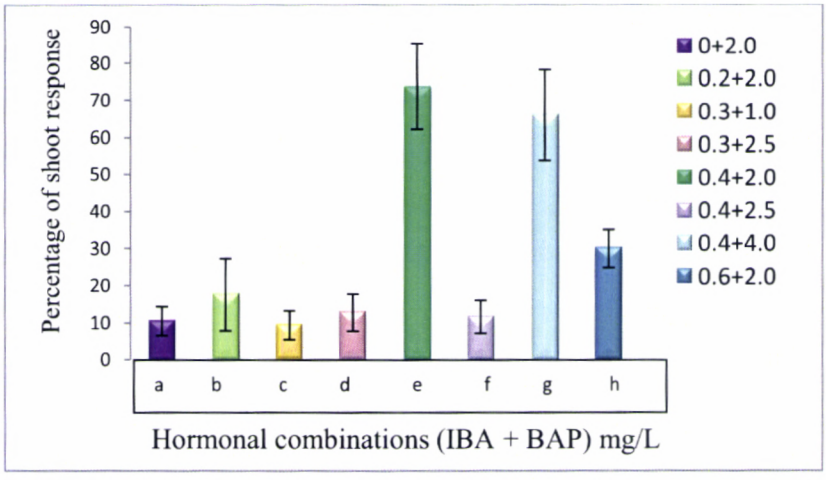


Figure 2.14: Effect of hormonal combinations on percentage of shoot response. Values represent the means ± SE.

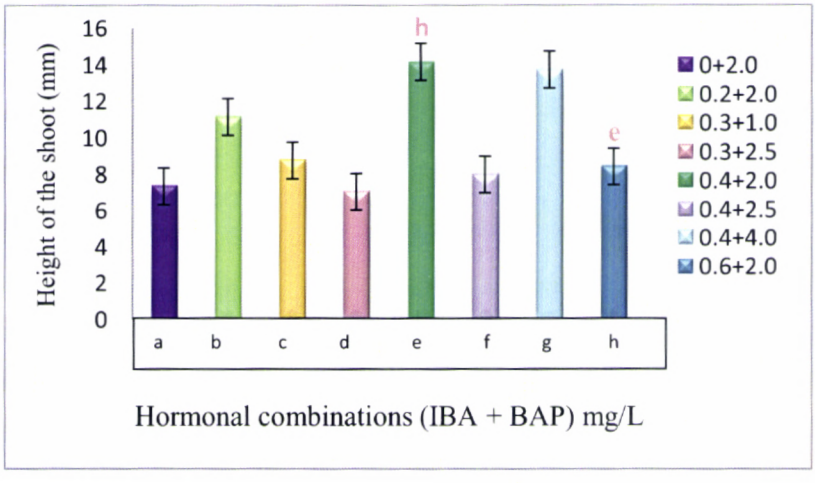


Figure 2.15: Effect of hormonal combinations on height of the shoot. Values represent the means ± SE. Letters above the bars indicate significant difference ($p<0.05$) based on Tukey's *Post hoc* test.

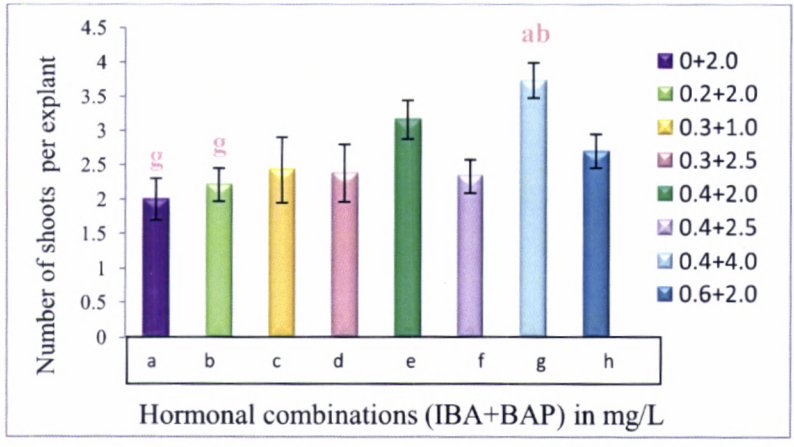


Figure 2.16: Effect of hormonal concentrations on multiple shoots induction. Values represent the means ± SE. Letters above the bars indicate significant difference ($p<0.05$) based on Tukey's *Post hoc* test.

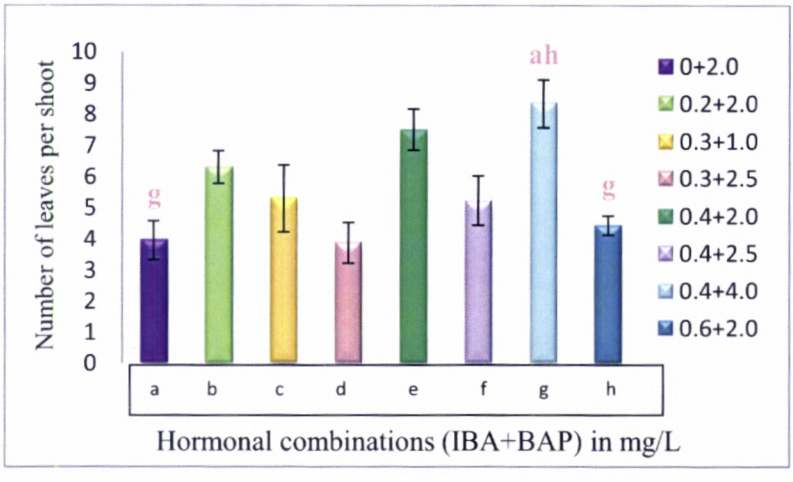


Figure 2.17: Effect of hormonal combination on number of leaves per shoots. Values represent the means ± SE. Letters above the bars indicate significant difference ($p<0.05$) based on Tukey's *Post hoc* test.

II.3.4. NODULAR CALLUS FORMATION FROM LEAF DISC CULTURE USING THIDIAZURON (TDZ)

Nodular calli were observed within 4-6 weeks in the media MS with different concentrations of TDZ and IBA. Morphological variation could be seen in different combinations. Light to dark green friable callus induced in various combinational treatments on the leaf discs. Intervening callus, small globular structures, resembling somatic embryos were started to appear from the lower surface of the leaf discs (Figure 2.22). These structures further developed when transferred into shoot elongation media. All the explants in the media augmented with 0.3 mg/L IBA and 2.5 mg/L TDZ, 0.4 mg/L IBA and 2.5 mg/L TDZ, and 0.4 mg/L IBA and 3.0 mg/L TDZ showed callogenesis. The data presented in Figure 2.18, showed that the MS supplemented with the above combinations are found to be most suitable for the initiation and development of shoot buds. The combination 0.4 mg/L IBA and 2.5 mg/L TDZ showed significant variation ($p<0.05$) in percentage of response with all other combinations except 0.3 mg/L IBA and 1.5 mg/L TDZ, 0.2 mg/L IBA and 2.0 mg/L TDZ, 0.3 mg/L IBA and 2.0 mg/L TDZ, 0.4 mg/L IBA and 2.0 mg/L TDZ, 0.3 mg/L IBA and 2.5 mg/L TDZ and 0.4 mg/L IBA and 4.0 mg/L TDZ (Appendix 2.c).

The percentage of shoot bud initiation (Figure 2.19) was higher (86.6%) in the 0.4 mg/L IBA and 2.5 mg/L TDZ and the combination showed significant variation ($p<0.05$) with all other combinations (Appendix 2.d). The number of shoot buds were found to be higher in 0.4mg/L IBA and TDZ 2.5 mg/L (Figure 2.20) and it showed significant variation ($p<0.05$) with all other combination except 0.3 mg/L IBA and TDZ 1.5 mg/L (Appendix 2.e). The maximum frequency of shoot elongation was only 48.6% in 0.4 mg/L IBA and TDZ 2.5 mg/L (Figure 2.21). The combination showed significant variation ($p<0.05$) in the number of roots with all the other combinations tried (Appendix 2.f). The media which provided with 1.0 and 2.0 mg/L TDZ failed

to show callus induction in the absence of IBA. But 3.0 mg/L TDZ supplement showed less amount of callus formation without nodules Shoot formation could be observed in 7th week after inoculation. Shoot apex with vascular tissue seen in safranin stained sections, when examined under microscope (40 X) (Figure 2.23).The length of the shoots and number of leaves were very less for plantlets formed.

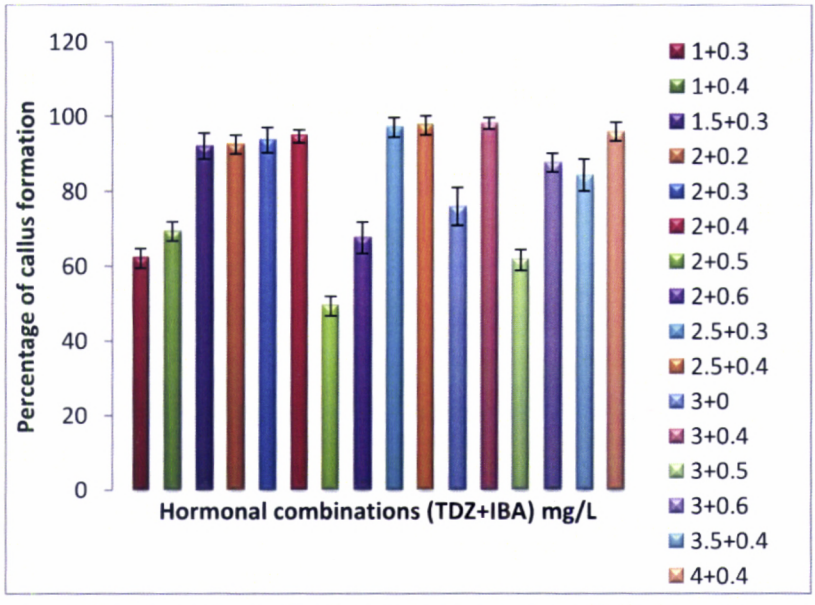


Figure 2.18: Effect of thidiazuron (TDZ) on percentage of callus induction from leaf disc culture. Values represent the means ± SE.

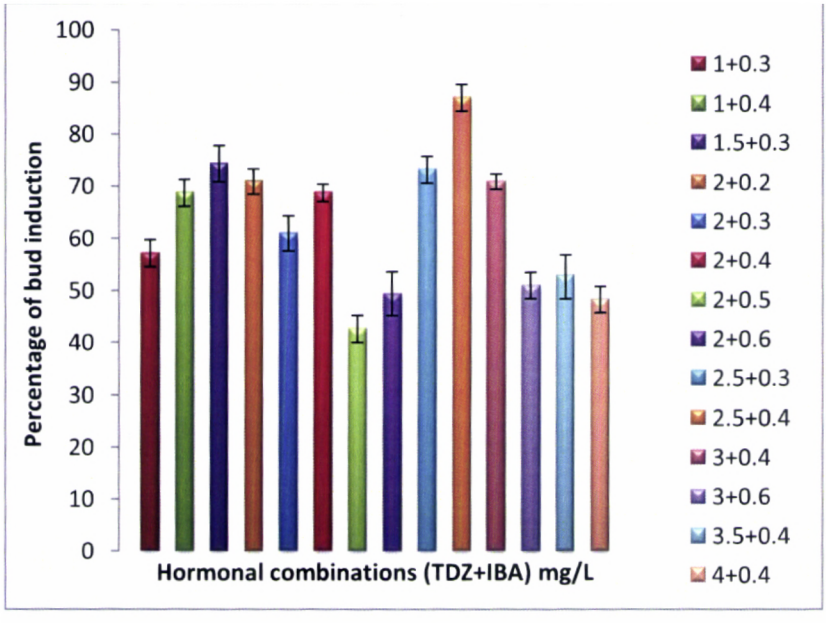


Figure 2.19: Effect of thidiazuron (TDZ) on percentage of bud induction from leaf disc culture. Values represent the means ± SE.

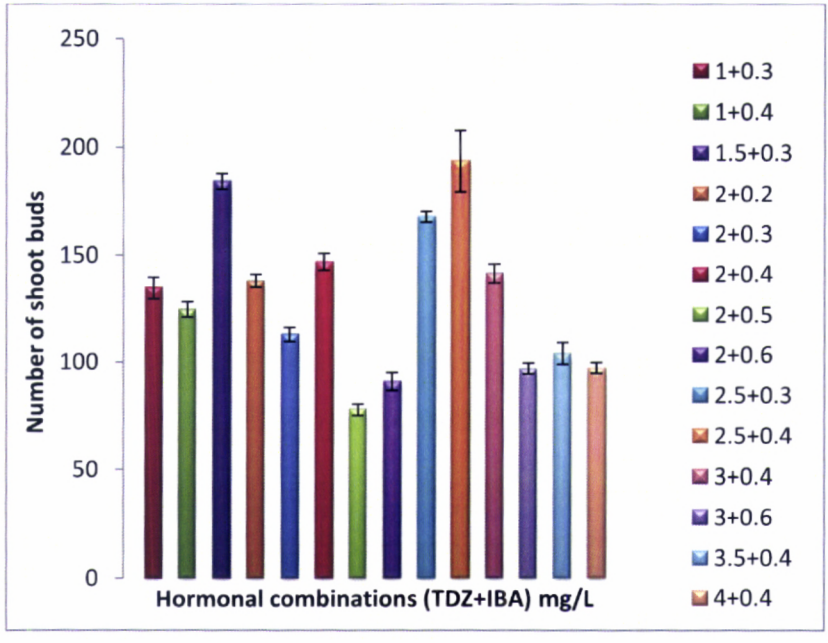


Figure 2.20: Effect of thidiazuron (TDZ) on number of shoot buds formed from leaf disc culture. Values represent the means ± SE.

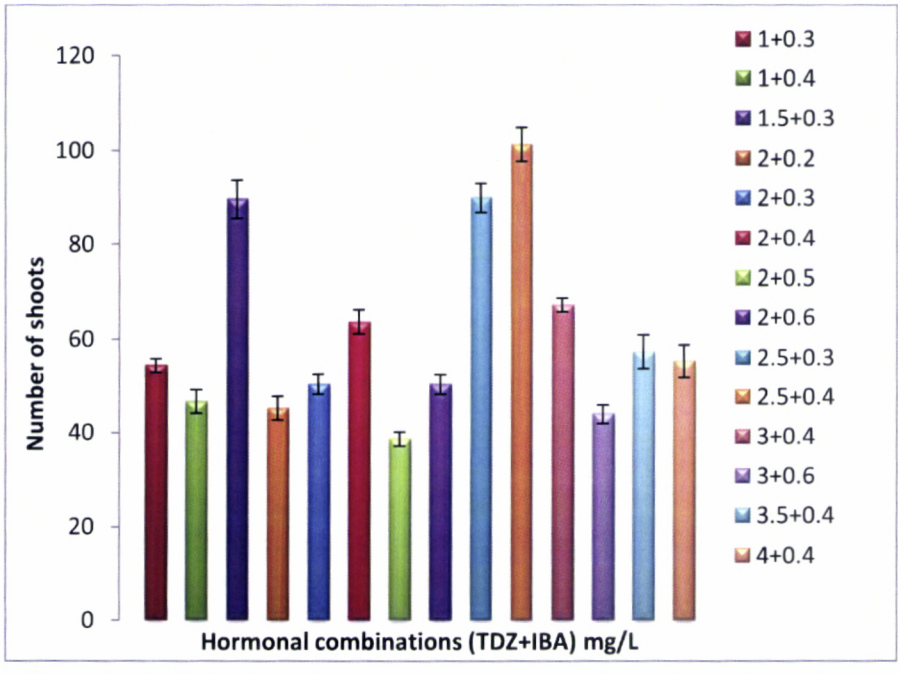


Figure 2.21: Effect of thidiazuron (TDZ) on number of shoot formed from leaf disc culture. Values represent the means ± SE.

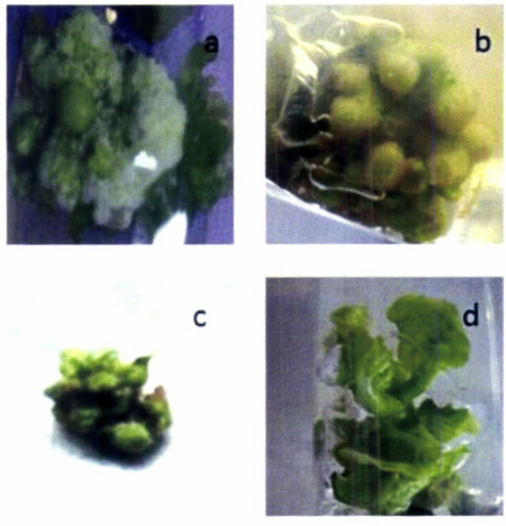


Figure 2.22. Shoot buds from the *Jatropha curcas* leaf discs in MS basal media with Thidiazuron. (a) Nodular calli (b) Shoot bud induction (c) Shoot initiation (d) Regenerated shoots

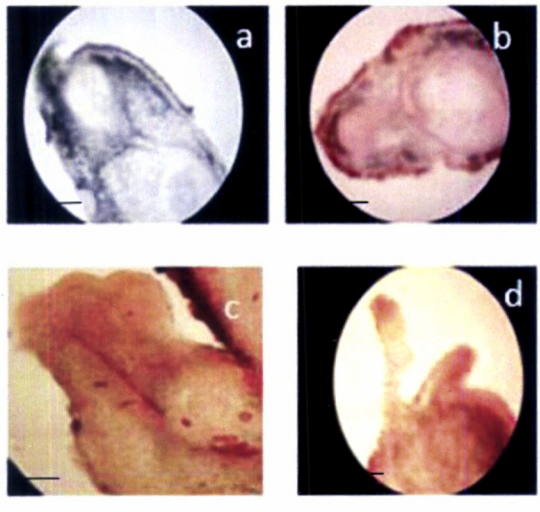


Figure 2.23: Safranin stained sections under light microscope (40X) (a) and (b) nodules formed in Thidiazuron supplemented media, (c) and (d) Shoot bud initiation (Bar represents 10μm)

II.3.5. ROOT INDUCTION

Root induction was found to be low in hormone supplemented medium. After 20 days of culture in MS medium with 0.2 mg/L IBA, root formation could be observed but it was not consistent, instead they produced callus at the basal cut portions of the shoots (Figure 2.24a). There was no response when 1.0 mg/L BAP supplemented along with the same. Same combination (0.2 mg/L IBA) when given with ½ strength MS media, the percentage of response was 23.3 whereas there was no root formation when ½ strength MS was fortified with 0.3 mg/L IBA. The phytohormones fortified medium showed swelling of the basal cut portion of the shoots and callus formation with very low frequency of root formation. Plant growth regulator free MS media of half strength showed root initiation faster (5days) (Figure 2.25) than in Water agar medium (7days) (Figure 2.24b and c) with 70.0% root induction. But the percentage of root response (76.7) and number of roots per shoots (2.4 roots) were more in Water agar-0.8% treatment (Figure 2.26 and 2.27).

II.3.5.1. Statistical analysis

MS supplemented with 0.2 mg/L IBA shows significantly high variation in percentage of root response ($p<0.001$), when compared to ½ strength MS without PGR and water agar, however the former does not show any significant variation ($p>0.05$) with ½ strength MS augmented with 0.2 mg/L IBA. Water agar medium shows significantly high variation when compared to MS and ½ strength MS with 0.2 mg/L IBA. There is no significant variation between rhizogenesis percentage in water agar and ½ MS basal medium. A slight variation could be observed in number of roots formed in water agar medium compared to MS with 0.2 mg/L IBA (2.4), ½ MS with 0.2 mg/L IBA (1.3) and ½ strength MS basal medium (1.6). There was no significant variation between the number of roots formed in the treatments MS with 0.2 mg/L IBA, ½ strength MS with 0.2 mg/L IBA, ½ strength MS basal medium.

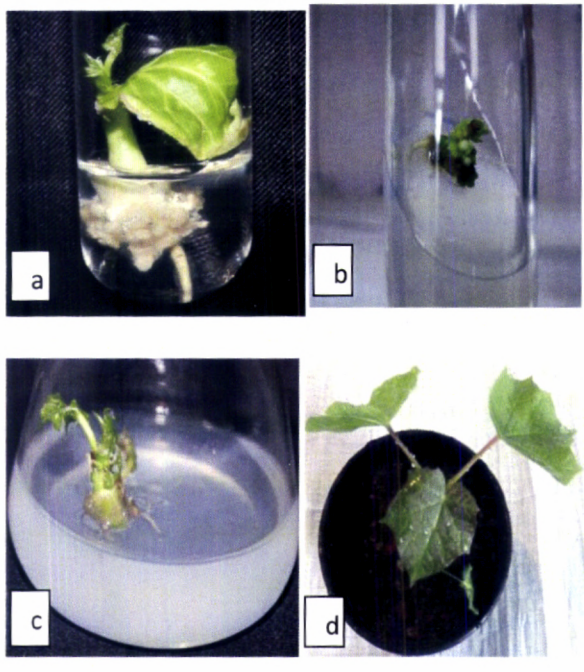


Figure 2.24: (a) Root induction in half strength MS with 0.2mg/L IBA (b) and (c) Rooted plantlet in 0.8% water agar medium (d) Hardened plant

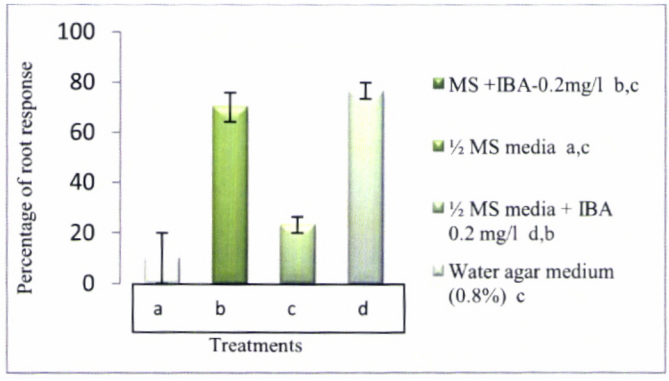


Figure 2.24: Effect of different treatments on percentage of root induction. Each treatment was replicated three times. Values represent the means ± SE. Letters indicate significant differences ($p<0.05$) based on Tukey's *Post hoc* test.

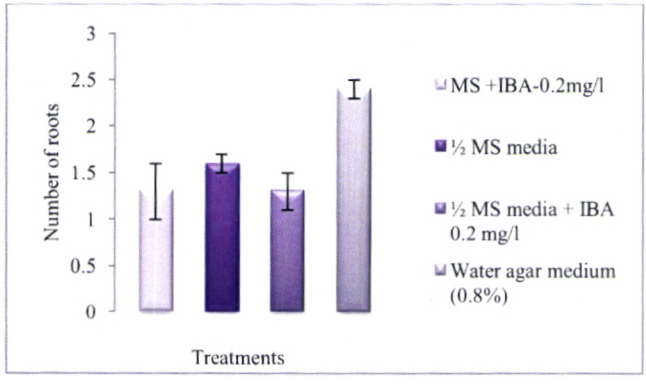


Figure 2.25: Effect of different treatments number of roots formed in the *Jatropha curcas*. Each treatment was replicated three times. Values represent the means ± SE. No significant difference ($p<0.05$) based on Tukey's *Post hoc* test.

II.3.6. ACCLIMATIZATION AND TRANSFER OF ROOTS

The regenerated plantlets transferred into the shadow house did not show any variation in their morphology and growth characteristics when compared with the donor plant (Figure 2.24d). The survival rate of the plantlets were 75.0±12.5% and 61.9± 8.2% respectively for the plantlets from water agar medium and ½ strength MS basal medium

II.3.7. MITOTIC INDEX STUDIES IN CALLUS CULTURES OF *JATROPHA CURCAS* MAINTAINED ON DIFFERENT HORMONAL COMBINATIONS

The sample harvesting time between 8.00 – 8.30 am on 3rd day of subculturing was found to be best for mitotic index studies. For metaphase arrest the 0.4% Colchicine for 4 h at room temperature found to be the best. Microscopic observation of the callus reveals that small cells have dense cytoplasm and large cells have vacuolated cytoplasm. Shape of cells varies from spherical to elongate. Hypotonic treatment of the sample callus, made the microscopic examination more clear, since it helped in the swelling of the cells. Different mitotic stages observed under 100X magnification (Figure 2.26). Fixation and maceration were found to be very critical steps in slide preparation. In MS medium with 2 mg/L BAP, the percentage of the cells in interphase were found to be high, compared to all other phases (Figure 2.27 a). In media supplemented with 0.2 mg/L IBA and 2 mg/L BAP, the percentage of the cells in later phases of mitosis were found to be lower (Figure 2.27 b), while for the medium with 0.3 mg/L IBA and 1.0 mg/L BAP, the proportion of cells in metaphase was found to be comparatively higher (Figure 2.27 c). Callus proliferation was faster in media with 0.3 mg/L IBA and 2.5 mg/L BAP with higher percentage of the cells in metaphase (Figure 2.27 d). When the hormonal concentration was 0.4 mg/L IBA and 2.0 mg/L BAP, the proportion of the cells in anaphase and telophase was found to be the highest contributing to a high value mitotic index (Figure 2.27 e) compared to that of 0.4

mg/L IBA and 2.5 mg/L BAP (Figure 2.27 f). The proportion of the cells in anaphase and telophase were lower in the callus from media with MS and IBA 0.4 and BAP 4 mg/L (Figure 2.27 g). The hormonal combination 0.4 IBA mg/L and 2.5 mg/L BAP gave callus with the same proportions of the cells in interphase and metaphase. The effect of hormonal composition on the mitotic index was compared (Figure 2.28). Media with hormonal composition 2 mg/L BAP showed the lowest mitotic index, while the highest mitotic index was seen in 0.4 mg/L IBA and 2 mg/L BAP.

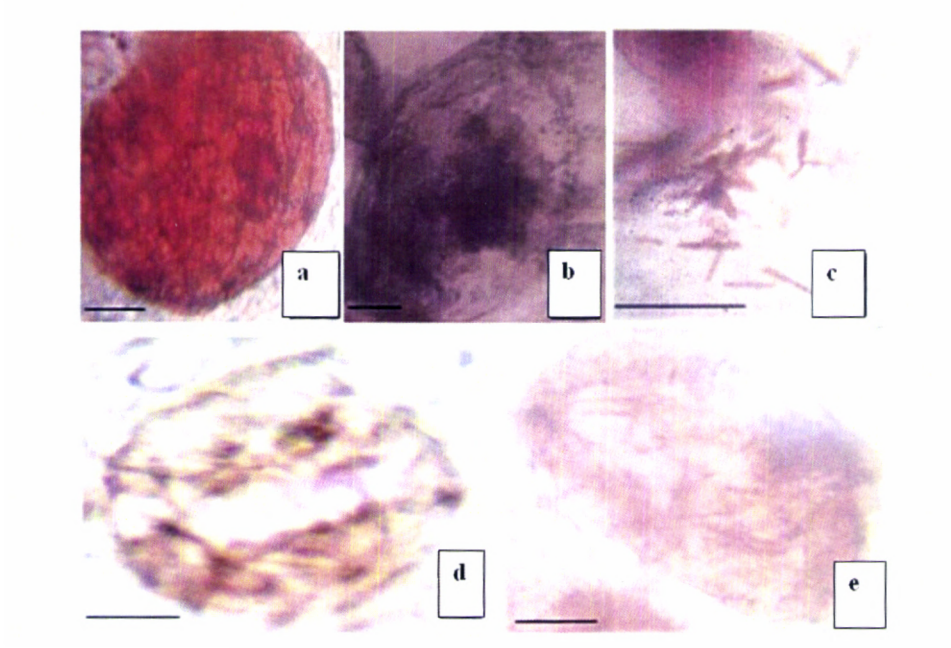


Figure 2.26: Photomicrographs showing mitotic stages under 100 X, a: Interphase, b: Prophase, c: Metaphase, d: Anaphase, e: Telophase (Bar represents 10μm)

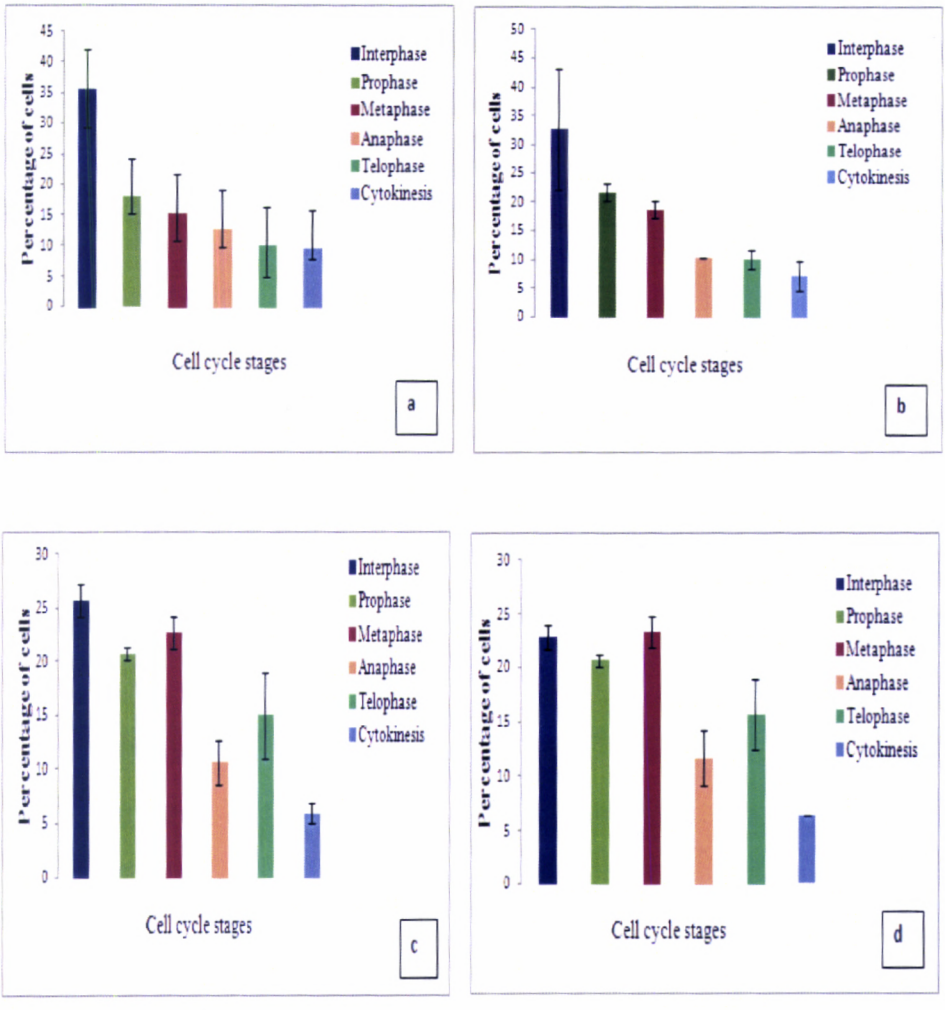


Figure 2.27 a-d: Effect of different auxin (IBA) and cytokinin (BAP) combinations on cell proliferation on MS medium supplemented with (a) 2.0 mg/L BAP (b) 0.2 mg/L IBA and 2.0 mg/L BAP (c) 0.3 mg/L IBA and 1.0 mg/L BAP (d) 0.3 mg/L IBA and 2.5 mg/L BAP. Values represent the mean ± SE.

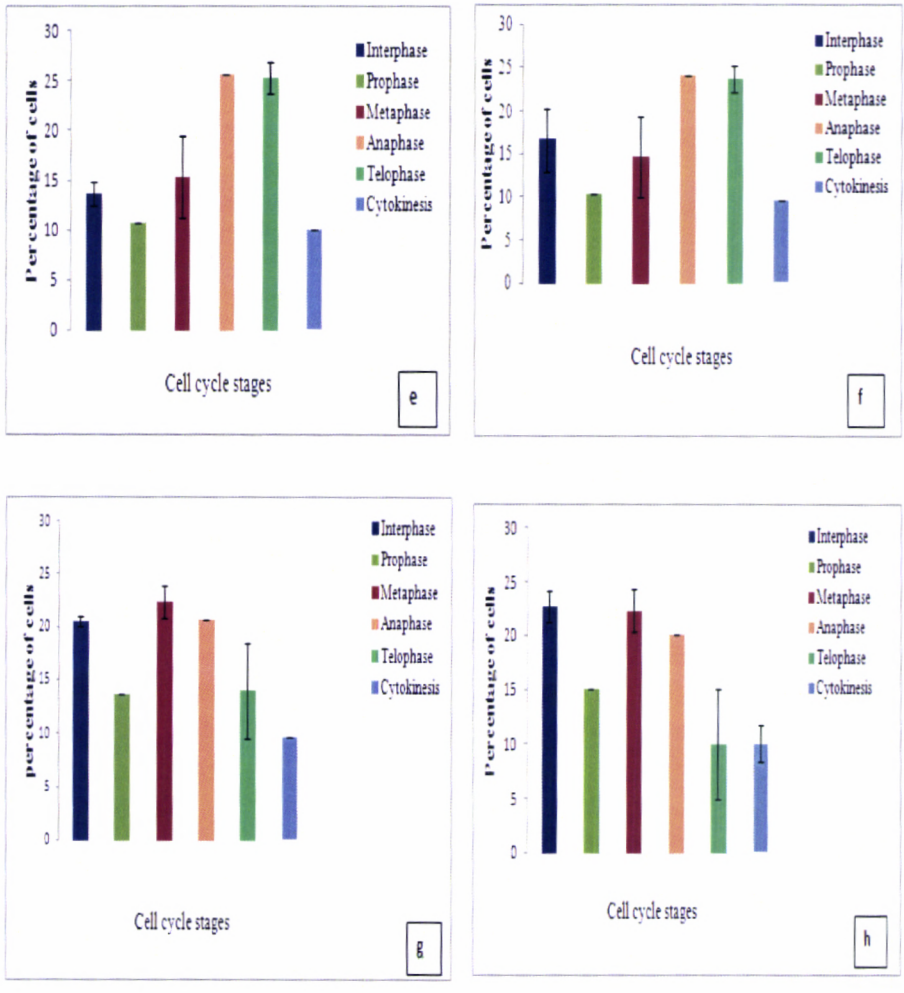


Figure 2.27 e-h: Effect of different auxin (IBA) and cytokinin (BAP) combinations on cell proliferation on MS medium supplemented with (e) 0.4 mg/L IBA and 2.0 mg/L BAP (f) 0.4 mg/L IBA and 2.5 mg/L BAP (g) 0.4 mg/L IBA and 4.0 mg/L BAP (h) 0.6 mg/L IBA and 2.0 mg/L BAP. Values represent the mean ± SE.

There was no significant difference ($p>0.05$) between the number of cells in telophase and cytokinesis in callus, when treated with 2.0 mg/L BAP and 0.6 mg/l IBA and 2.0 mg/L BAP. Hormonal combination 0.2 mg/l IBA and 2.0 mg/L BAP seemed to be given significant variation in the number of cells in each phases except number of cells in anaphase and telophase. Significant variation in the cell number in all the stages observed in the callus treated by the combination 0.3 mg/L IBA and 1.0 mg/L BAP whereas there was no significant variation ($p>0.05$) between interphase and metaphase cells count under the combination 0.3 mg/l IBA and 2.5 mg/L BAP. The combinations, 0.4 mg/l IBA and 2.0 mg/L BAP and 0.4 mg/l IBA and 2.5 mg/L BAP not showed significant variation between cell count in anaphase and telophase and prophase and cytokinesis. No significant variation ($p>0.05$) observed between cells count in interphase and metaphase under the combination 0.4 mg/l IBA and 4.0 mg/L BAP and 0.6 mg/l IBA and 2.0 mg/L BAP. 0.4 mg/l IBA and 4.0 mg/L treatment also not showed significant variation ($p>0.05$) between cell number in the phases, prophase and telophase, interphase and anaphase, and metaphase and anaphase. Other than these, number of cells in each phases varied significantly ($p<0.05$) each other in all the eight combinations.

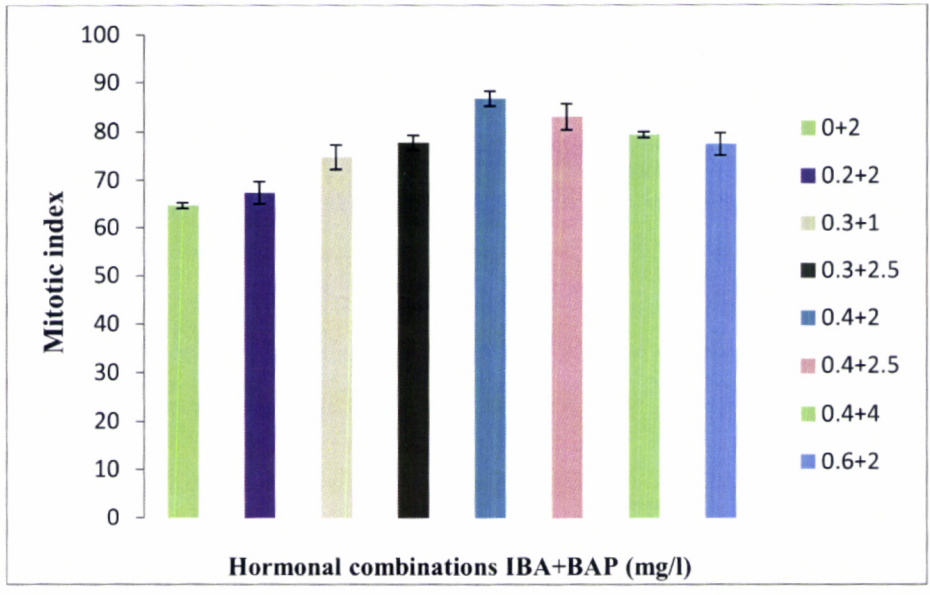


Figure 2.28: Effect of different hormonal concentration (IBA and BAP) on mitotic index in the *J. curcas* leaf disc callus. Values represent the means ± SE.

High mitotic indices of the media MS augmented with 0.3 mg/l IBA and 2.5mg/L BAP, 0.4 mg/L IBA and 2.0 mg/L BAP, 0.4 mg/L IBA and 2.5 mg/L BAP and 0.4 mg/L IBA and 4.0 mg/L BAP indicates its efficiency to pass the cells into exponential phase faster. Low mitotic index of the callus formed in the rest of the media shows that it will move the sigmoidal growth pattern of the callus towards right. There was no significant variation ($p>0.05$) in mitotic index between the combinations 0.3 mg/l IBA and 2.5 mg/L BAP and 0.6 mg/l IBA and 2.0 mg/L BAP whereas all other combinations showed significant variation ($p< 0.05$) each other.

II. 4. DISCUSSION

J. curcas is a latex producing shrub which makes it recalcitrant for handling in tissue culture (Shrivastava and Banerjee, 2008). Nevertheless during this study, plantlets with well developed shoots were produced through direct and indirect means from nodal and leaf explants respectively.

II.4.1. Hormonal combination of 1.0 mg/L BAP and 0.4 mg/L GA_3 was found to be the best for getting direct shoot regeneration from nodal explants

In the present study, the nodal culture responded maximum in MS medium with 1.0 mg/L BAP and 0.4 mg/L GA_3 (60%). Several studies support the role of GA_3 in shoot induction. Moallem *et al.* (2012) could obtain direct regeneration of *Rosa canina* in 0.25 mg/L BAP and 1.5 mg/L GA_3 combination. In a study by Canlih and Kazaz (2009) when combination of two hormones GA_3 and BAP was used; higher shooting percentage was observed compared to when BAP alone was used in proliferation of *R. hybryda.* The efficiency of GA_3 and BAP in breaking dormancy was reported by Rout *et al.* (1990).

Maharana *et al.* (2012) reported that higher shoot induction was achieved in 8 µM BAP for nodal explants of *J. curcas*. Satya and Rao (2009) also reported that the regeneration on MS supplemented with 3 mg/L BAP, from nodal explants of *J. curcas*. Axillary shoot buds were induced from seven month old nodal explants on MS medium supplemented with Kinetin, BAP, and TDZ along with Adenine sulphate (Koona *et al.* 2011).

Sugla *et al.* (2007) developed a protocol for the micropropagation of *Pongamia pinnata* from cotyledonary nodes derived from axenic seedlings. Murashige and Skoog (MS) medium supplemented with 7.5 µM Benzylaminopurine (BAP) induced up to 6.8 shoots per node with an average shoot length of 0.67 cm in 12 days. Incorporation of 2.5 µM gibberellic acid (GA_3)

in the medium during the first subculture after establishment and initiation of shoot buds significantly improved the shoot elongation.

Plants produced by direct organogenesis may exhibit greater genetic stability than those produced from callus (D'Amato, 1975). The availability of highly efficient regeneration protocol without the intervening callus phase is highly desirable for *Agrobacterium tumefaciens* mediated genetic transformation (Dan *et al.*, 2010). Therefore, the present investigation which showed direct shoot regeneration could be useful for mass production of true to type plants.

II.4.2. Nodal explants were better for direct regeneration than leaf and petiole explants

The explants source has been proved to be an important factor for *in vitro* growth and development of plant species, affecting callus induction and adventitious bud induction as well as shoot regeneration (Asghari *et al.*, 2012). In the present study it was observed that nodal explants were more appropriate than petiole and leaf disc explants for direct regeneration. The major problem observed during the study of nodal culture was that they were more prone to contamination. The availability of the explants was also a drawback of nodal culture. The different responses of the explant types are probably due to the endogenous hormonal balance in plant tissues (Grattapaglia and Machado, 1998).

Statistical analysis revealed that there were significant differences between the three tested explants of *Ocimum basilicum*, cotyledon explants, nodal and hypocotyls (Asghari *et al.*, 2012). These differences in the three explants can be explained by changes in the levels of endogenous hormones and the expression of genes encoding hormone receptors, as proposed by Close and Gallagher (1989). In a study by Maharana *et al.* (2012) nodal explants were found to be superior to leaf explants in direct regeneration of *J. curcas*. Internode explants showed higher frequency of shoot regeneration and number of shoots per explants than the leaf

and petiole explants and this could be attributed to mature and more vascular tissue in the internodes (Dhital *et al.*, 2010).

II.4.3. Highest frequency of callus induction was in MS medium with 1.5 mg/L BAP and 0.4 mg/L IBA

Callus induction is a prerequisite for large scale secondary metabolite production, adventitious improvement and for genetic engineering. Growth regulators are important factors, which can selectively influence the genes to trigger differentiation of cells in culture (Thorpe and Stefania, 1981; Thorpe, 1983). The texture of callus varies according to the nature of cytokinin and the auxin: cytokinin ratio (Martin *et al.*, 2003).

In this study, the morphology of the callus and percentage of response was found to vary depending on the exogenous growth regulators. Fast growing, compact callus could be induced from MS medium, supplemented with various combinations of IBA and BAP. The compactness of the callus increased with IBA concentration. The response of BAP was found to be better than Kinetin. The highest callus induction from petiole explants was in MS medium with 0.6 mg/L IBA and 2.0 mg/L BAP and from leaf explants best callus response was observed in 0.4 mg/L IBA and 1.5 mg/L BAP.

Biradar *et al.* (2012) reported callus induction from *J. curcas* using apical shoot tip and axillary leaf as explants on MS medium supplemented with various growth regulators *viz.* Kn, BAP and IAA. Weida *et al.* (2003) reported callus mediated Shoot-bud induction from *J. curcas* on Murashige and Skoog's (MS) medium supplemented with 1.0 mg/L IBA and 0.5 mg/L BAP.

II.4.4. Adenine sulphate does not have an effect on callus induction.

Adenine sulfate in combination with cytokinin and auxin has been used in tissue culture of many plant species (Zapartan, 2001). The introduction of adenine sulfate in the culture medium in flower bulb species has stimulated callus

differentiation in some combinations and of bulbs in other (Zapartan *et al.*, 2000). A study by Bantawa *et al.* (2009) demonstrated that the bavistin has stronger cytokinin-like activity than adenine sulphate. However, in the present study, it was found that adenine sulphate has no effect on callus induction.

II.4.5. Leaf explants were better for callus induction than petiole explants

Cell competence in the course of shoot bud regeneration is controlled by various internal factors (Ovecka *et al.*, 2000). Kim *et al.* (2001) suggested that the shoot formation ability of explants is related to the *in vivo* level of endogenous auxin and cytokinin and that the differential response to different cytokinins may be caused by the chemical and/or structural differences. In the present study leaf explants responded higher than the petiole explants. The frequency of callus induction was higher from leaf explant than from the internode and petiole explants in potato cultivars (Dhital *et al.*, 2010). Similar observation has been reported that the frequency of callus induction from leaf explants was higher than from petiole and internodal explants of *Fragaria ananassa* (Passey *et al.*, 2003). In a study by Dale and Deambrogio (1979) callus quality was found to be varying with the explants from the meristem tip, leaf sheath and mesocotyl, and highest from the root, immature embryo and mature embryo in *Hordeum vulgare*.

II.4.6. Callus induced from leaf explants was heterogeneous

Microscopic observation revealed that callus cells differ in size and appearance. Most cells in callus were spheroid and compactly arranged while others were uneven and loosely arranged. In a study by Saikia *et al.* (2012) in *A. Malaccensis* callus tissue obtained from leaf explants showed that the cellular composition was heterogeneous ranging from small cells with dense cytoplasm to large cells with vacuolated cytoplasm. The friable callus cells were loose with globular, oval, slightly elongated, beaked or comma in shape. The suspension cultures of *J. curcas* comprised mainly of round, densely cytoplasmic starch

containing cells with distinct nuclei (Soomro and Memon, 2007). In a study by Datta and Conger (1999) in Switchgrass (*Panicum virgatum* L.) the culture was found to be comprised mainly of small, round densely cytoplasmic starch containing cells with distinct nuclei. Some cells were large elongated and highly vacuolated with sparse cytoplasm

II.4.7. Indirect regeneration occurred from leaf discs in MS medium with 0.4 mg/L IBA and 2.0 mg/L BAP combination

Vegetative plant parts especially leaves are desirable explants for *in vitro* cultures because plant regeneration from these explants would preserve the genetic identity of the parent genotype (Sridhar and Naidu, 2011).

Depending on species or cultivars, the most important achievement obtained in the propagation of many plant materials through tissue cultures has been frequently based on the successful adjustment of the type and combination of plant growth regulators (Tran, 1981; Murashige, 1990; Gurel and Gulsen, 1998). BAP and Kn alone were unable to induce shoot buds and shoot bud induction decreased with increase in concentration of Kn. In the present study, MS medium supplemented with IBA in combination with BAP was found to be best for shoot induction. Thidiazuron (TDZ) and IBA in combination with BAP were best suited for adventitious shoot bud induction from leaf explant of *J. curcas* in the study of Deore and Johnson (2008). Kaul *et al.* (2010) reported that shoot bud formation was accompanied with callus formation on medium supplemented with 2.46 µM IBA and 13.3 µM BAP. Prabhakaran and Sujatha (1999) found that IBAin combination with BAP in MS medium helped in elongation of shoots developed from leaf discs of *J. curcas* and its hybrids.

In the present investigation a lower concentration of IBA and a slightly higher concentration of BAP was best for shoot induction. The higher concentrations of auxins are generally inhibitory to morphogenesis; and substitution with an appropriate auxin-cytokinin ratio is essential for obtaining

proper shoot and root primordia. The study by Rajore and Batra (2005) showed that the 0.5 mg/L IBA and 1.5 mg/L BAP combination is very effective in shoot bud differentiation. In our study the best response (73.83%), was obtained in MS basal media with 0.4 mg/L IBA and 2.0 mg/L BAP combination.

BAP is considered as one of the most useful cytokinins for achieving the multiplication and micropropagation of plants (Stfaan *et al.*, 1994). There are several reasons for preferred use of BAP as cytokinin. Slow degradation of BAP is one of the reasons and it can be autoclaved without losing its activity. However Dhar and Joshi (2005) and Baker *et al.* (1999) have reported the combinational effect of auxin and cytokinin to be fruitful for axillary and apical bud sprouting. According to Grattapaglia and Machado (1998), cytokinins such as BAP and Kn are very effective in promoting proliferation. Cytokinins participate in the regulation of many plant processes that induce callus cell division in the presence of auxin, leading to bud or root formation directly on the explant or from calli (Taiz and Zeiger, 2002).

II.4.8. Thidiazuron (TDZ) induced nodular structures from leaf disc

TDZ, a substituted N-phenyl urea (Ricci *et al.*, 2001) is able to induce diverse morphogenic responses ranging from tissue proliferation to adventitious shoots and somatic embryo formation. In direct regeneration system, thidiazuron (TDZ), shows cytokinin-like activity and is known to be more active than zeatin for stimulating the growth, differentiation and organogenesis, especially in direct shoot regeneration, when added to a tissue culture medium at a low concentration (Sujatha *et al.*, 2005; Deore and Johnson, 2008; Kumar and Reddy, 2010; Kumar *et al.*, 2010). In the present study TDZ resulted in abnormal growth and similar result was reported by Sujatha *et al.* (2005) in nontoxic *J. curcas* leaf explant. The ability of TDZ to induce high shoot regeneration in woody plant tissues has been reported (Huetteman and Preece, 1993; Meng *et al.*, 2004). In strawberry-leaf tissues, TDZ in combination with IBA induced high-frequency shoot induction

(Landi and Mezzetti, 2006). The present study further supports the role of TDZ on shoot-bud induction from *J. curcas* leaf discs.

In the media MS with different concentrations of TDZ and IBA, small globular nodular structures started to appear from the lower surface of the leaf discs. These structures further developed into shoot buds. Whereas media in which IBA was absent failed to show nodular structures. In the study of induction of direct somatic embryogenesis it is showed that auxin alone in MS media was unable to give embryogenesis (Ramasamy *et al.*, 2005). Similarly, Kumari *et al.* (1998) found the requirement of cytokinin in addition to auxin to induce somatic embryogenesis in *Terminalia arjuna.* Similar observation was made for the species *Psoralea corylifolia* (Sahrawat and Chand, 2002).

Apart from cytokinin like activity, TDZ has been suggested to be a modulator of the endogenous auxin levels. There is experimental evidence that TDZ stimulates de novo synthesis of auxins by increasing the levels of IAA and its precursor, tryptophan (Murthy *et al.*, 1995). Increases in endogenous auxin, cytokinin, and ethylene have been seen in response to TDZ treatment (Murthy *et al.*, 1995). As a consequence, TDZ has been shown to be useful for rapid plant regeneration in several recalcitrant species through organogenesis (Malik and Saxena, 1992). In the present study, it was observed that TDZ induces multiple shoot buds at high-frequency from leaf discs.

II.4.9. Hormone free medium and water agar was found to be more efficient than hormone supplemented medium for root induction

In the current study root induction was found to be low in IBA supplemented medium and showed callus formation. Low concentrations of auxin increased root growth, but high concentrations prevented growth of roots. The reduced growth of roots is attributed to high ethylene concentration in high concentrations of auxin (Taiz and Zeiger, 2002). Regenerated shoots could be rooted on growth regulator-free MS medium also by Qin *et al.* (2004).

In the case of shoot tip culture of *J. curcas*, IBA (0.5 - 5.0 mg/L) incorporated singly in MS was found suitable for root induction whereas in combination with IAA it caused callus formation (Rajore and Batra 2005). Efficient rooting was obtained in 0.1 mg/L IBA containing medium as compared to other auxins (Sujatha *et al.*, 2005)

In the current study percentage of root response and number of roots per shoots were more in water agar treatment. The root induction may be due to the nutritional stress. Adventitious rooting in *Thryptomene ericaea* was induced by culturing elongated shoots in 0.7% (w/v) water-agar supplemented with 1.0 mM indole-3-butyric acid (IBA) for 48 hours then transferring the shoots to hormone free basal medium for 8 weeks. Under these conditions, a rooting frequency of 85% was achieved (Ainsley and Lee, 2010). The treatment of almond shoots resulted in the best rooting when shoot insertion done for 12 h into water-agar (0.6% w/v) with 1.0 mM IBA, followed by 2 weeks in basal medium without auxin but with 100.0 µM phosphatidylglycerol (Ainsley *et al.*, 2001). Iron stress induced root hair formation has been reported by Schmidt and Schikora (2001) in Arabidopsis.

II.4.10. Mitotic index was highest in callus induced on MS medium with 0.4 mg/L IBA and 2 mg/L BAP

Many aspects of role of auxin in cell division processes depend on the multiple controls exerted by auxin on cell division and cell expansion (Rechenmann, 2010). The mitotic index of the callus was very low in the MS medium supplemented with only BAP (2.0 mg/L). This observation emphasizes the role of auxin in growth and of cytokinin in cell division (De, 1992). Auxins and cytokinins, such as BAP and IAA, are the main growth regulators that act to control cell division and tissue differentiation (Feher *et al.* 2003).

NAA stimulates cell elongation at concentrations that are much lower than those required to stimulate cell division. In contrast, 2, 4-D promotes cell division

but not cell elongation (Campanoni and Nick, 2005). Complexity of possible pathways of auxin and cytokinin action dependent on species, genotypes and tissues studied by Coenen and Lomax (1997) and reported that cytokinin influences the level of auxin.

It was demonstrated that cytokinin stimulates the removal of the phosphate, activation of the enzyme kinase complex and the synchronous entry of the cells into mitosis (Zhang *et al.*, 1996). In this study, hormonal concentration 0.4 mg/L IBA and 2 mg/L BAP supplemented in MS medium showed highest mitotic index and is found to be best in callus proliferation for *J. curcas* leaf explants. The plant growth regulators interfere in cell cycle control and may lead to genetic variability (Bairu *et al.*, 2011). Growth regulators can influence somaclonal variations during the culture phase through their effects on cell division (Gould, 1984), the degree of disorganized growth (Karp, 1982), and selective proliferation of specific cell types (Ghosh and Gadgil, 1979).

CHAPTER III: EVALUATION OF VARIATION IN CALLUS AND REGENERANTS USING MOLECULAR MARKERS

III.1. REVIEW OF LITERATURE

III.1.1. EVALUATION OF SOMACLONAL VARIATION

Somaclonal lines may be more variable than breeder lines for most agronomic yield components and quality characters (Hanson *et al.*, 1994). In order to evaluate somaclonal variants, several strategies have been used based on evaluation of morphological traits, cytogenetic analysis (numerical and structural variation in the chromosomes), or studying molecular and biochemical markers (Rani *et al.*, 1995). Somaclonal lines may be more variable than breeder lines for most agronomic yield components and quality characters (Hanson *et al.*, 1994).

III.1.1.1. CYTOLOGICAL ANALYSIS

Raja *et al.* (1992) reported that chromosomal rearrangements and numerical variation of chromosomes to play a major role in inducing somaclonal variation. Karyological analysis can reveal significant chromosomal changes such as alteration of ploidy level as well as structural rearrangements (Brown *et al.*, 1993). Chromosomal alteration and ploidy changes are highlighted by cytogenetic analysis, including chromosome counting and flow cytometry. Cytometry has been used to identify the particular characteristics of somaclonal variation in *Vitis vinifera* (Faure and Nougarède, 1993, Kuksova *et al.*, 1997., Leal *et al.*, 2006) and *Citrus lemon* (Orbovic *et al.*, 2008).

A thorough cytological analysis on different embryogenic and non-embryogenic oil palm (*Elaeis guineensis*) calli was performed and chromosome

instability studied as a possible cause of somaclonal variants in regenerated plants by Lucia *et al.* (2011). The chromosomes segregation and the occurrence of haploid gamete like cells could be observed. The intrinsic instability of oil palm chromosomes was also verified. In a study by Suri and Saini (2007) plantlets obtained through seedling derived embryonic callus of *Chlorophytum borivilianum* showed high level of morphological and cytological variation, which increased with the increase in age of cultures. However karyology analysis cannot reveal alteration in specific genes or in small chromosome arrangements (Masoud *et al.*, 2008).Variegated leaves were also observed occasionally. High level of variation in the leaf size, stomata number and epidermal cell size were observed.

III.1.1.2. VARIATION ANALYSIS USING MOLECULAR MARKERS

Various molecular markers have also been used to study somaclonal variation, such as isoenzymes (Saker *et al.*, 2000), RAPD (Random Amplified Polymorphic DNA) markers (Hashmi *et al.*, 1997; Piola *et al.*, 1999; Virscek-Marn *et al.*, 1999; Caboni *et al.*, 2000; Saker *et al.*, 2000; Nas 2004), rDNA genes (Anderson *et al.*, 1991; Brettel *et al.*, 1986; Breiman *et al.*, 1987), chloroplast DNA (Kawata *et al.*, 1995), Internal transcribed spacer 1 (ITS-1) sequences (Banerjee *et al.*, 2012), AFLP (Amplified fragment length polymorphisms) markers (Polanco and Ruiz, 2002; Prado *et al.*, 2010; Prado *et al.*, 2007; Chuang *et al.*, 2009) and Inter-simple sequence repeats (ISSR) (Albani and Wilkinson, 1998; Jain 2001; Nookaraju and Agrawal, 2012; Pathak and Dhawan, 2012; Patzak, 2003).

III.1.1.2.1. Random Amplified Fragment Length Polymorphisms (RAPD)

The random amplified polymorphic DNA (RAPD) approach developed by Williams *et al.* (1990) using arbitrary primers has become the best known variant of this prototype of PCR-based DNA profiling. RAPD polymorphism results from either a nucleotide base change that alter the primer binding site, or from an insertion or deletion within the amplified region. Polymorphism usually results in the presence or absence of amplification products from a single locus (Tingey and Tufo, 1993). RAPD markers are not typically influenced by environmental conditions or the developmental stage (Belaj *et al.*, 2001; Deshwall *et al.*, 2005).

Suggestions have been made that somaclonal variants tend to be concentrated in "hotspots". Thus, for example, heavy metal stress has been shown to direct DNA methylation to specific loci (Aina *et al.*, 2004). Hypervariable RAPD products have been observed among the tissue culture regenerants of rye (Cassells and Roche, 1994). Supporting the concept of non-random induction of (epi) mutations in tissue culture is the observation that a number of demethylation and *de novo* methylation events, as well as sequence changes, were inherited by all (or at least 90%) of the regenerants in *Hordeum vulgare* L. (Bednarek *et al.*, 2007).

Masoud *et al.* (2008) studied regenerated plants of the tree cotton cultivars and found significant differences in morphological characters like the length of shoots, number of leaves and in the number of RAPD loci identified as well as degree of polymorphic bands. Different sub cultures produced different level of genetic diversity in the cultivars of cotton. With an increase in the time period of sub-cultures an increase in the amount of genetic variation occurred in the regenerated plants.

III.1.1.2.2. Amplified Fragment Length Polymorphism (AFLP)

Detection of DNA polymorphism using AFLP markers in bulk samples have been reported in several plant species (Kolliker *et al.*, 2001; Papa *et al.*, 2007). Both morphological characteristics and amplified fragment length polymorphism (AFLP) markers were used to validate the genetic fidelity of field-grown *Echinacea purpurea* plants regenerated from leaf explants (Chuang *et al.*, 2009).

III.1.1.2.3. Simple Sequence Repeats (SSR)

SSRs, also known as Anchored Simple Sequence Repeats (ASSRs), are a class of molecular markers aiming at amplifying the interspaces between two SSR sequences (Zietkiewicz *et al.*, 1994). Microsatellites are simple sequence repeated (SSR) motifs present in the plant nuclear DNA that are locus specific, highly polymorphic and co-dominant. This latter property, moreover, allows the discrimination between homozygotes and heterozygotes (Sefc *et al.*, 2001). ISSR has unique advantages over other molecular markers though it has some limitations. It doesn't need any genomic information of the target species, which is extremely important in a preliminary investigation. It uses small amount of template DNA and it can be rapidly conducted, which is an efficient way to detect diversity of a species in a short time (Semagn *et al.*, 2006).

In fact the inter-simple sequence repeats are regions lying within the microsatellite repeats, have a high capacity to reveal polymorphism and offer great potential to determine intra-genomic and inter-genomic diversity as compared to other arbitrary primers, like RAPDs (Zietkiewicz *et al.*, 1994). ISSR permits detection of polymorphisms in inter-microsatellite loci, using a primer designed from dinucleotide or trinucleotide simple sequence repeats (Wu *et al.*, 1994; Zietkiewicz *et al.*, 1994).

The use of ISSRs as genetic markers in crop plants has been demonstrated in several agricultural crop species, including wheat (Vaillancourt *et al.*, 2008), rice (Reddy *et al.*, 2009) and maize (Domenyuk *et al.*, 2002). ISSRs have been extensively applied in cultivar and species identification (He *et al.*, 2007), and diversity/polymorphism evaluation of germplasm (Nan *et al.*, 2003). ISSR markers could reveal complex, species-specific patterns from a variety of eukaryotic taxa, and also intraspecies polymorphisms. Thus ISSR markers are applicable for taxonomic and phylogenetic analyses (Zietkiewicz *et al.*, 1994). SSR primer MsCir 74 showed polymorphism in directly regenerated plantlets of sugar cane NIA-98 (Khan *et al.*, 2008).

The primers with poly (GA) n and poly (AG) n motifs produced more polymorphism than any other motif, while primers with (AT) n, (GT) n and other motifs did not give any amplification (Gupta *et al.*, 2008). Somewhat similar results were also reported by Ajibade *et al.* (2000). Nagaoka and Ogihara (1997) evaluated applicability of ISSR as a genetic marker system in wheat and found that ISSR primers produce more reliable and reproducible bands when compared with RAPD primers. According to their study, ISSR primers produced several times more information than RAPD markers. The extent of band polymorphism greater than that of RAPDs. However Gupta *et al.*, (2008) reported that they obtained a high reproducibility for both RAPD and ISSR markers once the PCR conditions were well set up. RAPD markers were more efficient than ISSR assay, as they detected 84.26% polymorphism in *J. curcas* as compared with 76.54% for ISSR markers. This is in contrast to the results as obtained for several other plant species like wheat (Nagaoka and Ogihara, 1997) and vigna (Ajibade *et al.*, 2000).

III.1.1.2.4. Direct Amplification of Minisatellite DNA (DAMD)

Minisatellites are tandemly repeated DNA regions of eukaryotic genomes, many of which showed high levels of allelic length variation due to the deference in the number of repeated units (Jeffreys *et al.*, 1985). These loci are highly informative genetic markers that have been used extensively in many areas of genetics. Direct amplification of minisatellite DNA markers (DAMD-PCR) technique, introduced by Heath *et al.* (1993) has been explored as a means of generating DNA probes useful for detecting polymorphism. DAMD-PCR clones can yield individual-specific DNA fingerprinting pattern and thus have the potential as markers for species differentiation and cultivar identification (Somers *et al.*, 1996).

A study on mini satellite DNA is important as it is highly unstable having highest mutation rate. The DAMD-PCR technique offers several advantages to other molecular methods (Bebeli *et al.*, 1997). DAMD method is robust, rapid and applicable to any genome and has been routinely used for diversity and biosystematic studies in plants such as betelvine, mulberry, papaya and *Murraya* species (Shirish *et al.*, 2008).

DAMD PCR based approach was used to identify accession specific DNA markers and study genetic relationships between and within 15 accessions corresponding to 11 species in genus Capsicum (Ayse *et al.*, 2009). Direct amplification of minisatellite DNA by PCR (DAMD PCR) was used to amplify and subsequently clone several fragments of DNA from crucifer species (Somers and Demmon, 2002). Their study suggested that dispersed, genome-specific probes can be isolated using DAMD PCR for further studies in the minisatellite regions. Eleven primer pairs were proposed by Ranade *et al.* (2008) for probing polymorphic DNA sequences (RAPD) and minisatellites (DAMD) in Jatropha.

Plants of *Vitis vinifera* regenerated from callus cultures showed polymorphism using methylation-sensitive restriction enzymes (Popescu *et al.*, 2002). Somaclonal variation induced by UV-C radiation in potato was revealed by 5 out of 28 random primers and also ISSR4 primer after PCR amplification of DNA (Ehsanpour *et al.*, 2007). Genotype clustering can be detected by UPGMA analysis and ultimately revealed by a dendogram (Ranade *et al.*, 2008).

III.1.1.3. MORPHOLOGICAL AND BIOCHEMICAL ANALYSIS

Callus-derived somaclonal variation based on morphological and biochemical parameters have been reported in strawberry (Popescu *et al.*, 1997), sunflower (Pajevic *et al.*, 2004), *Capsicum annuum* (Anu *et al.*, 2004), tea (Jibu *et al.*, 2006), Dieffenbachia (Shen *et al.*, 2007), Sugarcane (Rajeswari *et al.*, 2009), wheat (Shah *et al.*, 2009), rice (Park *et al.*, 2010), Caladium (Thepsithar *et al.*, 2010), St. Augustinegrass (Li and Bruneau, 2010), olive (Yari and Farahani, 2011; Yari *et al.*, 2011) and anthurium (Winarto *et al.*, 2011). The rate of changes varies not only in response to tissue culture conditions, but also among species and even among cultivars of the same species (Karp, 1982). Proteins and isozymes have been used widely as markers for identifying cultivars and characterising somaclonal variation in many fruit species (Eastman *et al.*, 1991). After acclimatisation the water retention capacity of *in vitro*-formed apple leaves was lesser than *in vivo*-formed leaves (De, 2002).

Morphological diagnosis is relatively simple, but it requires laborious field experiments and it is time-consuming (Nwauzoma *et al.*, 2002; Piagnani *et al.*, 2008). Additionally, morphological characteristics are frequently affected by the developmental stage and environment (Hussain *et al.*, 2009). Bajaj *et al.* (1986) have observed range of morphological variations in cereals and grasses. During growth and

development processes, many plants present changes in their enzymatic expression. The use of isoenzymes as markers is well documented and their genetically defined variants have evidenced their importance for evaluating genetic variability in some crops, such as rice, barley, maize, bean, potato, wheat and soybean (Cevallos, 2000; Chamberlain *et al.*, 1996). Isoenzyme markers provide an appropriate method to detect genetic changes. However, these markers are susceptible to ontogenetic variation and are limited in number (Masoud *et al.*, 2008).

Isozyme analysis has been used to assess genetic fidelity in *Citrus* plants regenerated through organogenesis and somatic embryogenesis (Carini and Pasquale, 2003). Qualitative and quantitative traits such as the amount of free fatty acids, unsaponifiables, acid number and carbon residues show a wide range of variation. This indicates that the oil quality in regenerant is dependent on the interaction of environmental and genetic factors (Kaushik *et al.*, 2007).

Significantly high curcumin, oleoresin and volatile oil contents (%) were observed in somaclonal variants of *Curcuma longa* L. when compared to the normal regenerants and control plant (Roopadarshini and Gayatri, 2012). Phytochemical content found to be more in regenerants in results reported by Mathur *et al.* (1989) in aromatic crops and Ravindra *et al.* (2004) in rose scented geranium. Kaushik *et al.* (2001) observed that the biochemical changes in seeds and pods during the development of *J. curcas*. Developing capsules were harvested from the 17th day after anthesis in 10 days interval until maturity. The seed protein profiles, obtained through polyacrylamide gel electrophoresis, on *Jatropha* species, *J. curcas, J. heterophylla, J. gossypifolia,* and *J. panduraefolia,* showed distinct protein profile. Homology could be found between Jatropha and Castor. Similarity indexes indicated *J. gossypifolia* as being the closest to Ricinus. Apparently, Ricinus and Jatropha, despite their

morphological resemblances, are distinct genera, although perhaps with a common ancestor now extinct (Sathaiah and Tummala, 1985).

III.2.1. PLANT MATERIAL

The callus grown in media supplemented with 8 different hormonal combinations (S1-S8) (Table 3.1), leaves of the mother plant (S9) and plantlets regenerated from the corresponding medium were analysed for similarity to the mother plant, *Jatropha curcas* L., TBGT-70000. Prior to DNA isolation, the callus and regenerated plants were washed in distilled water to remove agar and the leaves were rinsed and kept under running tap water with a drop of tween 20.

Table: 3.1 Different hormonal combinations used for callus mediated regeneration and variation studies

S. No.	IBA+ BAP Conc.(mg/L) in MS media
1	0.0 +2.0
2	0.2 +2.0
3	0.3 +1.0
4	0.3+.2.5
5	0.4+2.0
6	0.4+2.5
7	0.4 +4.0
8	0.6+2.0

III.2. 2. DNA ISOLATION

DNA isolation method by Doyle and Doyle (1987), using CTAB yielded good quality DNA for PCR. The procedure is described below.

III.2.2.1. Solutions

An extraction buffer consisting of 2% CTAB (w /v), Tris HCl pH 8.0 (1M); EDTA pH 8.0 (0.5M); NaCl (4M) (Appendix 3.a) was prepared. 3M Sodium acetate solution (pH 5.2), Chloroform: Isoamylalcohol (24: 1), Phenol: Chloroform: Isoamylalcohol (25:24:1 v/v/v), Ethanol (70%) and TE buffer (Appendix 3.b) were the additional solutions required.

III.2.2.2. Preparation of buffer saturated phenol for DNA extraction

Water bath was maintained at 65°C. Bottle of phenol was kept in water bath to melt the crystals. Equal volume of 0.5M Tris HCl (pH 8.0) was added to phenol and mixed for 15 minutes. Poured the mixture to a separating funnel and allowed the two phases to separate. The bottom layer was allowed to pass through the separating funnel and rest was discarded. Equal volume of 0.1M Tris HCl (pH 8.0) was added to the phenol. Repeated the same procedure.

III.2.2.3. DNA isolation using CTAB

Freshly prepared CTAB buffer was preheated at 65°C. 1gm of the *Jatropha curcas* tissue sample was ground in 16 ml of CTAB buffer and homogenized. The ground tissue incubated at 65°C in a water bath for 30 minutes followed by incubation at room temperature. Equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1) was added and mixed thoroughly to form an emulsion. The aqueous layer was collected after centrifugation at 10,000 rpm for 10 minutes and transferred to a new 50 ml centrifuge tube using a cut tip. Phenol: chloroform: isoamyl alcohol extraction was repeated and the aqueous layer was collected in eppendorf tubes (1ml in each tube). 3M sodium acetate (pH 5.2) was added to the

aqueous phase and DNA was precipitated by the addition of 2/3rd volume of ice cold isopropanol and thoroughly mixed by inverting. The samples were kept for overnight incubation at -20°C. The solution was taken after incubation and centrifuged at 12,000 rpm for 10 minutes. The supernatant was decanted off and the pellet was washed with cold 70% ethanol. The DNA was further pelleted by centrifugation at 12,000 rpm for 10 minutes. The ethanol was decanted off and the pellet was air dried to remove all traces of ethanol and resuspended in 100µl TE buffer.

III. 2.2.4. Amount and purity of DNA

The yield of DNA extracted was measured using a UV Spectrophotometer (UV-1601 SHIMADZU) at 260 nm. The purity of DNA was determined by calculating the ratio of absorbance at 260 nm to that of 280 nm. DNA concentration and purity was also determined by running the samples in 1.0% Agarose gel, based on the intensities of band obtained by staining with (0.5 µg/ml) Ethidium bromide when compared with 250 bp DNA marker from Chromous Biotech (Appendix 3.c and d).

III.2.2.5. PCR Mix Composition

1X PCR Buffer (10mM Tris HCl pH 8.3; 50mM KCl)

1.5 mM $MgCl_2$

0.2 mM dNTP

0.5µM of single primer

1 U of *Taq* DNA polymerase (Sigma Aldrich)

50 ng of template DNA

Sterile distilled water

III.2.3.2. PCR Condition

Initial denaturation -5 minutes- at 94 ° C

Denaturation - 1 minute, at 92°C

Annealing - 1 minute at 35 °C

Extension -2 minutes - at 72°C

Final extension at 72°C for 10 minutes

PCR was performed for 39cycles in a thermal cycler Biorad MJ Mini thermal cycler. The PCR mix composition and conditions used were same for RAPD, ISSR and DAMD analysis unless specified separately. Reaction without DNA was used as negative control. Each reaction volume was 20µl.

III.2.3. PRIMERS FOR PCR AMPLIFICATION AND RAPD ANALYSIS

DNA extracted from *J.curcas* callus and regenerated plantlets were used for RAPD analysis with 35 oligonucleotide primers from Operon Technologies Inc. Almeda CA, USA (Table 3.2).

III.2.4. PRIMERS FOR PCR AMPLIFICATION AND ISSR ANALYSIS

DNA extracted from *J. curcas* callus induced in different hormonal combinations (Table: 3.1) and regenerated plantlets were used for ISSR analysis with 20 oligonucleotide primers from Operon Technologies Inc. Almeda CA, USA (Table 3.3). Reactions were carried out with 2.0 mM $MgCl_2$ providing annealing temperature of 55 °C for 2 minutes.

Table 3.2: Primers from Operon Technologies Inc. Almeda CA, USA used for RAPD analysis

S no	Primer	Sequence	GC%
1	OPA-01	CAGGCCCTTC	70
2	OPA-02	TGCCGAGCTG	70
3	OPA-03	AGTCAGCCAC	60
4	OPA-04	AATCGGGCTG	60
5	OPA-05	AGGGGTCTTG	60
6	OPA-08	GTGACGTAGG	60
7	OPA-18	AGGTGACCGT	60
8	OPA-11	CAATCGCCGT	60
9	OPA-13	CAGCACCCAC	70
10	OPB-11	GTAGACCCGT	60
11	OPB-15	GGAGGGTGTT	60
12	OPB-18	CCACAGCAGT	60
13	OPC-07	GTCCCGACGA	70
14	OPC-08	TGGACCGGTG	70
15	OPC-09	CTCACCGTCC	70
16	OPD-05	TGAGCGGACA	60
17	OPD-07	TTGGCACGGG	70
18	OPD-08	GTGTGCCCCA	70
19	OPD-12	CACCGTATCC	60
20	OPD-14	CTTCCCCAAG	60
21	OPE-01	CCCAAGGTCC	70
22	OPE-02	GGTGCGGGAA	70
23	OPE-05	TCAGGGAGGT	60
24	OPF-11	TTGGTACCCC	60
25	OPG-17	ACGACCGACA	60
26	OPH-18	GAATCGGCCA	60
27	OPJ-15	TGTAGCAGGG	60
28	OPK-01	CATTCGAGCC	60
29	OPK-12	TGGCCCTCAC	70
30	OPL-05	ACGCAGGCAC	70
31	OPL-14	GTGACAGGCT	60
32	OPM-12	GGGACGTTGG	70
33	OPP-04	GTGTCTCAGG	60
34	OPAL-11	GTCACGTCCT	60
35	OPAP-19	GTGTCTGCCT	60

Table 3.3: Primers from Operon Technologies Inc. Almeda CA, USA used for ISSR analysis

S. no.	Primer	Sequence	Number of base pairs	GC%
1	ISSR 1	ATA TAT ATA TAT ATA TT	17	00
2	ISSR2	AGA GAG AGA GAG AGA GT	17	47
3	ISSR 3	GAG AGA GAG AGA GAG AT	17	47
4	ISSR 4	CTC TCT CTC TCT CTC TT	17	47
5	ISSR 5	GTG TGT GTG TGT GTG TA	17	47
6	ISSR 6	TCT CTC TCT CTC TCT CA	17	47
7	ISSR 7	ACA CAC ACA CAC ACA CT	17	47
8	ISSR 8	TGT GTG TGT GTG TGT GA	17	47
9	ISSR 15	ACC ACC ACC ACC ACC ACC	18	67
10	ISSR 16	CCG CCG CCG CCG CCG CCG	18	100
11	ISSR 17	GGCGGCGGCGGCGGC GGC	18	100
12	ISSR 18	TGC TGC TGC TGC TGC TGC	18	67
13	ISSR 19	CTA GCT AGC TAG CTA G	16	50
14	ISSR 20	TGC ATG CAT GCA TGC A	16	50
15	ISSR 21	CTT CAC TTC ACT TCA	15	40
16	ISSR23	AGA GTT GGT AGC TCT TGA TC	20	45
17	ISSR u1	GGGTG GGGTG GGGTG	15	80
18	ISSR u2	GAA GAA GAA GAA GAA GAA	18	33
19	ISSR u3	GGAGA GGAGA GGAGA	15	60
20	ISSR u4	GAG AGA GA G AGA GAG AA	17	47

III.2.5. PRIMERS FOR PCR AMPLIFICATION AND DAMD ANALYSIS

DNA extracted from *Jatropha curcas* callus and regenerated plantlets were used for DAMD analysis with 5 primers from Operon Technologies Inc. Almeda CA, USA (Table 3.4). Concentration of $MgCl_2$ used was 2.5 mM. Annealing temperature was 55°C for 2 minutes.

Table 3.4: Primers from Operon Technologies Inc. Almeda CA, USA used for DAMD analysis

S. no.	Primer	Sequence	Number of base pairs	GC%
1	HVR	CCTCCTCCCTCCT	13	69
2	HBV	GGTGTAGAGAGGGGT	15	60
3	HVA	AGGATGGAAAGGAGGC	16	56
4	M13	GAGGGTGGCGGTTCT	15	67
5	33.6	AGGGCTGGAGG	11	73

III.2.6. AGAROSE GEL ELECTROPHORESIS

PCR products were electrophoresed on 2% (w/v) agarose gels, in 1X TAE Buffer at 120 V for 2hrs and then stained with Ethidium bromide (0.5 µg/mL). Gels with amplicons were visualized and photographed in Gel Documentation system (Syn gene).

III.2.7. DATA ANALYSIS

Each RAPD, ISSR and DAMD product was assumed to represent a single locus and data were scored for presence (1) and absence (0) of bands. Index of genetic variation was calculated and dendrograms were constructed through UPGMA

(Unweighted Pair Group Method with arithmetic mean) method using computer software POPGENE (ver. 1.32) of Yeh *et al.* (1997).

III.3.1. DNA ISOLATION

The DNA extracted from the callus samples from MS media with different hormonal combination S1-S8 (Table 3.1), regenerated plants from the corresponding calli and mother plant (S9) were of good quality with absorbance at 260/280 nm between 1.741-1.946 (Figure 3.1).

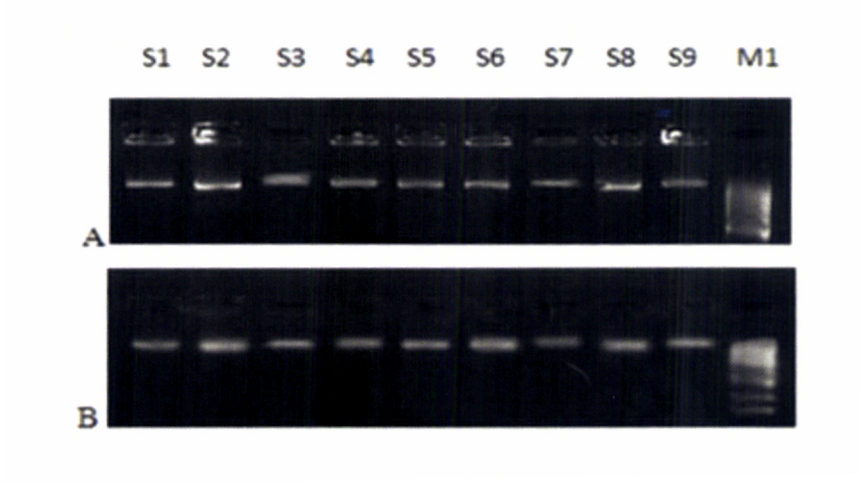


Figure 3.1: (A) Lanes S1 – S8: Genomic DNA obtained from callus from MS media with different hormonal combination (S1-S8) and Lane S9: Genomic DNA obtained from mother plant. (B) Lanes S1 – S8: Genomic DNA obtained from *in vitro* regenerantss from corresponding media (S1-S8) and Lane S9: Genomic DNA obtained from mother plant. Lane M1 – DNA marker Chromous Biotech (250bp), (See Table 3.1 for media combinations).

III.3.2. EVALUATION OF VARIATION IN CALLUS CULTURES AND REGENERATED PLANTS OF *J. CURCAS* USING RAPD MARKERS

III.3.2.1. RAPD Marker analysis in callus cultures

Of 38 decamer primers used for the RAPD analysis, 11 primers showed consistent band patterns. In total, 35 scorable bands were observed with the 11 primers (Table 3.5). The total number of amplicons produced per primer varied from 2 for OPA05, OPD07, OPE 2, OPM 12 and OPM12 to as many as 7 bands for OPE01 (Figure 3.2). The average number of bands per primer was 3.18. Out of 35 bands, 30 were polymorphic (85.71%). The average number of polymorphic RAPD bands was 2.72 per primer. Polymerase chain reaction with RAPD primers OPA05, OPA11, OPC08, OPE01and OPH-18 showed one monomorphic band in all samples (S1-S8 and in mother plant genomic DNA).

Table 3. 5: Primers used for RAPD analysis and the band characteristics obtained for the callus from different hormonal combinations and mother plant

Primers	Sequences	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (P%)	Total bands amplified
OPA05	AGGGGT CTTG	2	1	0	50.0	13
OPA11	CAATCG CCGT	3	2	1	66.6	23
OPC08	TGGACC GGTG	4	3	1	75.0	20
OPD07	TTGGCA CGGG	2	2	0	100	16
OPD08	GTGTGC CCCA	3	3	0	100	13
OPE01	CCCAAG GTCC	7	6	1	85.7	39
OPE02	GGTGCG GGAA	2	2	0	100.0	12
OPH18	GAATCG GCCA	4	3	1	75.0	22
OPK12	TGGCCC TCAC	3	3	0	100.0	17
OPL05	ACGCAG GCAC	3	3	0	100.0	13
OPM12	GGGACG TTGG	2	2	0	100.0	9

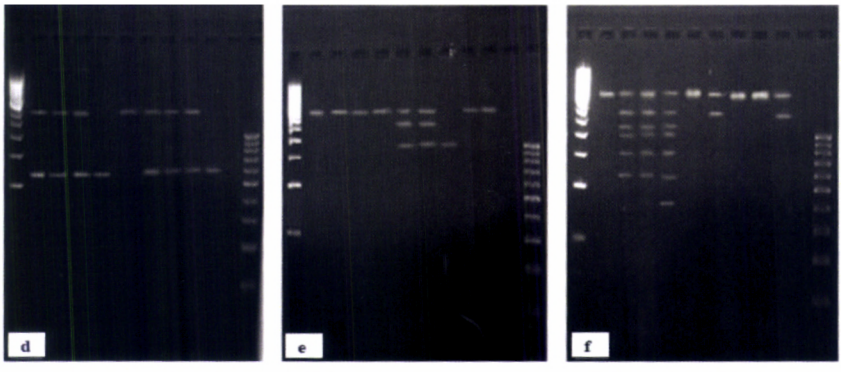


Figure 3.2: RAPD pattern generated by primer (a) OPA-05, (b) OPA-11, (c) OPC-08, (d) OPD-07, (e) OPD-08, (f) OPE-01. Lane M1 –DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from callus from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J. curcas*; Lane N: Negative control; Lane M2: DNA marker from Step Up Genei (100bp)

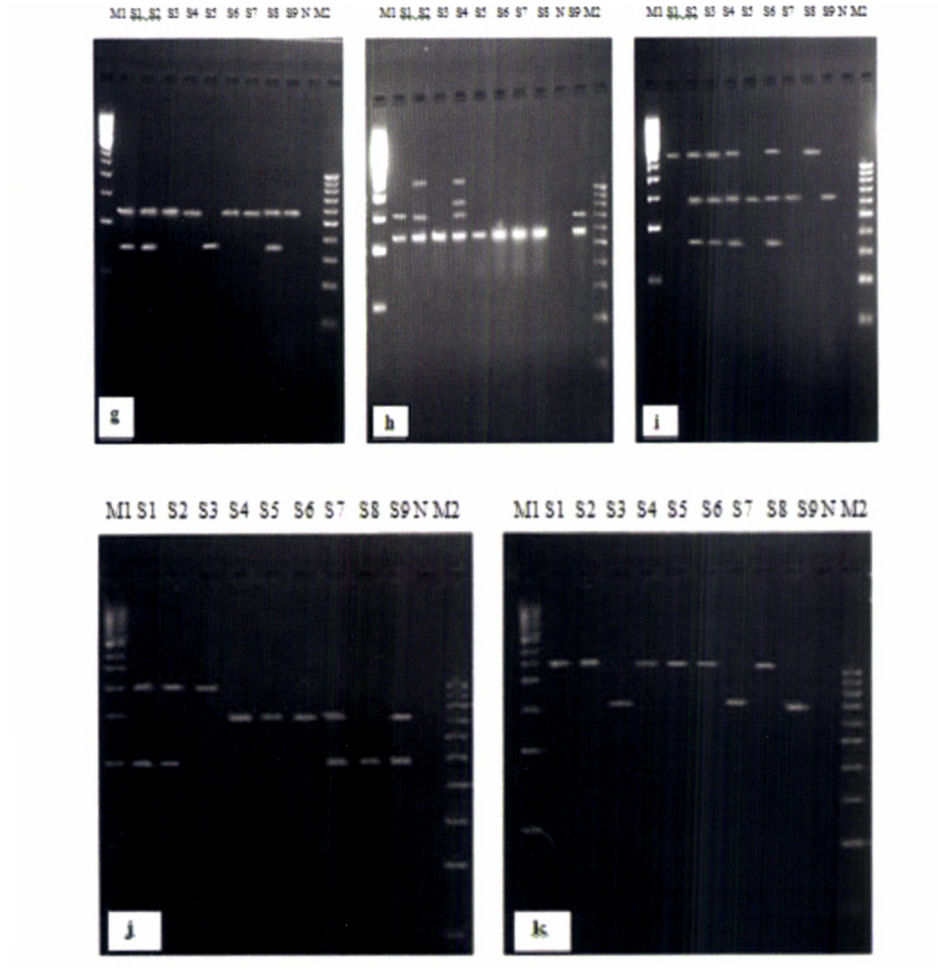
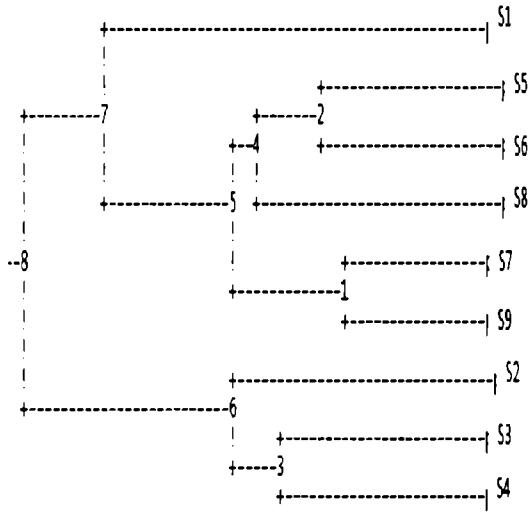


Figure 3.2: RAPD pattern generated by primer (g) OPE-02, (h) OPH-18, (i) OPK-12, (j) OPL-05 and OPM-12 (k). Lane M1 –DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from callus from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J. curcas*; Lane N: Negative control; Lane M2: DNA marker from Step Up Genei (100bp).



Between	And	Length
8	7	5.17598
7	pop1	25.75557
7	5	8.67462
5	4	0.61467
4	2	4.07447
2	pop5	12.39181
2	pop6	12.39181
4	pop8	16.46627
5	1	7.72037
1	pop7	9.36058
1	pop9	9.36058
8	6	13.58799
6	pop2	17.34356
6	3	3.36431
3	pop3	13.97924
3	pop4	13.97924

Figure 3.3: Dendrogram Based Nei's (1972) Genetic distance between the DNA samples of callus from MS media with different hormonal concentration and mother plant using UPGMA- Modified from NEIGHBOR procedure of PHYLIP Version 3.5. S1-S8 are the DNA samples of callus from MS media with different hormonal concentration, S9 Genomic DNA obtained from mother plant of *J. curcas*

Table 3.6: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between the DNA samples of callus from MS media with different hormonal concentration and mother plant from RAPD profile. S1-S8 are the DNA samples of callus from MS media with different hormonal concentration, S9 Genomic DNA obtained from mother plant of *J. curcas*

DNA samples	S1	S2	S3	S4	S5	S6	S7	S8	S9
S1	****	0.5854	0.4634	0.4634	0.5854	0.5122	0.5854	0.6585	0.6585
S2	0.5355	****	0.6829	0.7317	0.4634	0.4878	0.3659	0.6341	0.5366
S3	0.7691	0.3814	****	0.7561	0.5854	0.5610	0.4878	0.5610	0.6585
S4	0.7691	0.3124	0.2796	****	0.6341	0.6585	0.4878	0.6098	0.5610
S5	0.5355	0.7691	0.5355	0.4555	****	0.7805	0.6585	0.7317	0.6829
S6	0.6690	0.7178	0.5781	0.4177	0.2478	****	0.7317	0.7073	0.7561
S7	0.5355	1.0055	0.7178	0.7178	0.4177	0.3124	****	0.7317	0.8293
S8	0.4177	0.4555	0.5781	0.4947	0.3124	0.3463	0.3124	****	0.7073
S9	0.4177	0.6225	0.4177	0.5781	0.3814	0.2796	0.1872	0.3463	****

Considering the dendrogram (Figure 3.3) constructed from the pooled data, it is evident that the set of primers chosen for the study were able to detect the influence of growth regulators on the callus generated. Apparently the samples could be divided into two groups. Five samples viz., S5, S6, S7, S8 and S9 (motherplant) were genetically close to each other and hence formed a group. Another distinct group is formed by S2, S3 and S4. However S1 was found to be distinct from the two groups. Pairwise similarity computed for each method separately is given in Table: 6. Among the callus from different samples, the highest similarity (0.78) was between callus from hormonal combination 0.4 mg/L IBA and 2.5 mg/L BAP (S6) and 0.4 mg/L IBA and 2.0 mg/L BAP (S5), while the least similarity (0.36) was between the callus from MS supplemented with 0.4 mg/L IBA and 4.0 mg/L BAP (S7) and 0.2 mg/L IBA and 2.0 mg/L BAP (S2) (Table 3. 6). Similarly, the highest similarity with mother plant

(0.82) showed by the callus from hormonal combination 0.4 mg/L IBA and 4.0 mg/L BAP (S7) where S2 (callus from 0.2 mg/L IBA and 2.0 mg/L BAP) showed the least similarity (0.53) with mother plant.

III.3.2.2. RAPD Marker analysis in regenerated plants

Table 3. 7: Primers used for RAPD analysis and the band characteristics obtained for the regenerated plantlets from different hormonal combinations and mother plant

Primers	Sequences	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (P%)	Total bands amplified
OPA 05	AGGGGTCTTG	3	0	3	00.0	27
OPA 11	CAATCGCCGT	3	1	2	33.3	24
OPC 08	TGGACCGGTG	3	2	1	66.6	16
OPD 07	TTGGCACGGG	2	1	1	50.0	12
OPD 08	GTGTGCCCCA	3	2	1	66.6	20
OPE 01	CCCAAGGTCC	6	1	5	16.6	53
OPE 02	GGTGCGGGAA	2	0	2	00.0	18
OPH 18	GAATCGGCCA	2	0	2	00.0	18
OPK 12	TGGCCCTCAC	3	1	2	33.3	26
OPL 05	ACGCAGGCAC	2	1	1	50.0	16
OPM 12	GGGACGTTGG	2	1	1	50.0	10

There were 31 scorable bands generated by 11 primers (Table 3.7). The number of amplicons per primer ranges from 2 to 6 for different primers (Figure 3.4). The average number of bands per primer was 2.81. 67.74% polymorphism could be seen in 31 bands. The average number of polymorphic RAPD bands was 1.90 per primer.

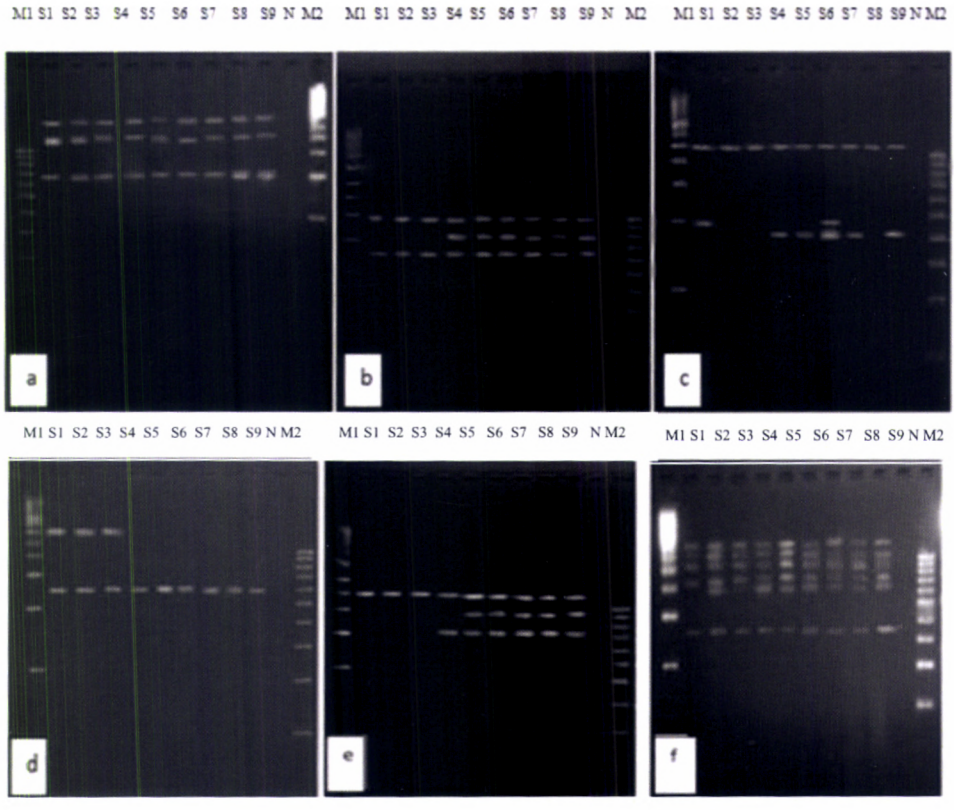


Figure 3.4: RAPD pattern generated by primer (a) OPA-05, (b) OPA-11, (c) OPC-08, (d) OPD-07, (e) OPD-08, (f) OPE-01. Lane M1 –DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from regenerants from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J.curcas*; Lane N: Negative control; Lane M2 –DNA marker from Step Up Genei (100bp) (See Table 3.1 for media composition)

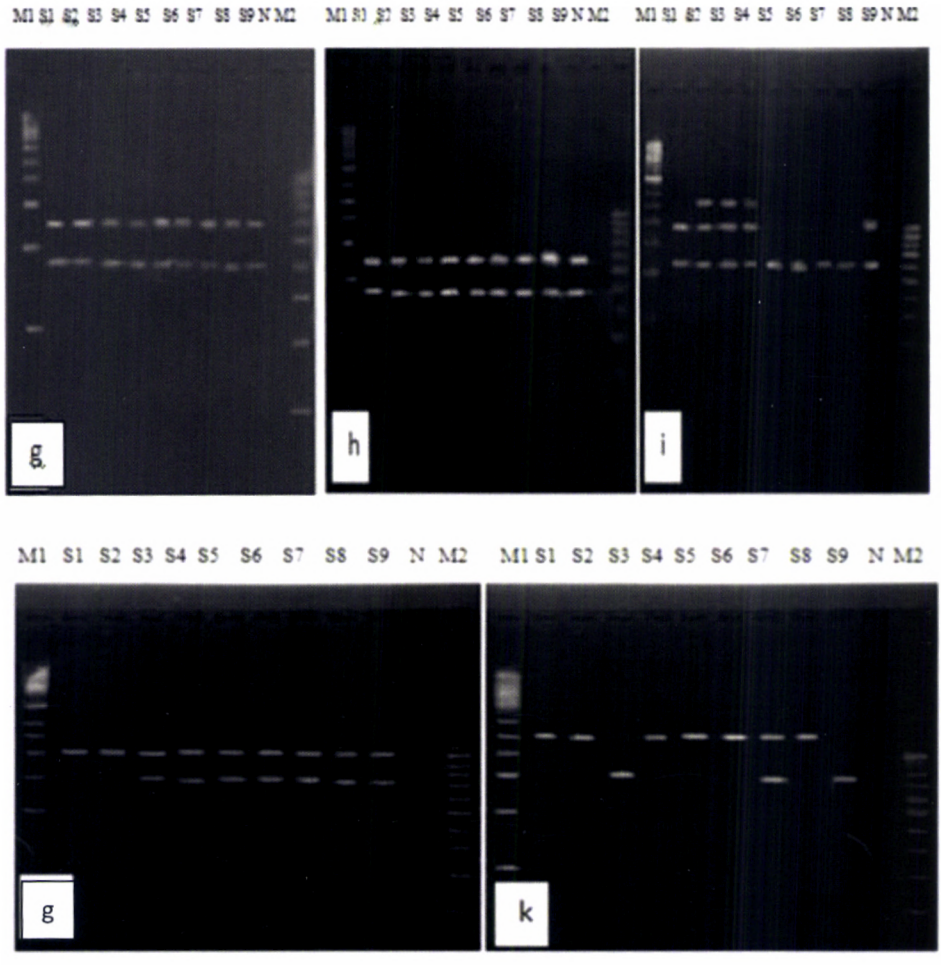
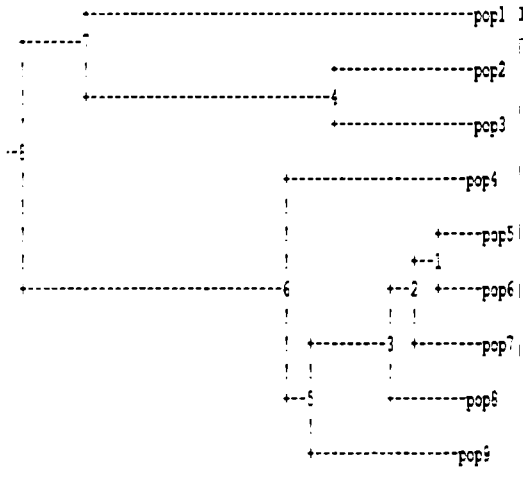


Figure 3.4: RAPD pattern generated by primer (g) OPE-02, (h) OPH-18, (i) OPK-12, (j) OPL-05 and OPM-12 (k). Lane M1 –DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from regenerants from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J. curcas*; Lane N: Negative control; Lane M2 –DNA marker from Step Up Genei.



Between	And	Length
8	7	2.10461
7	pop1	13.44703
7	4	8.52502
4	pop2	4.92200
4	pop3	4.92200
8	6	9.22598
6	pop4	6.32566
6	5	0.95014
5	3	2.69509
3	2	0.27325
2	1	0.81975
1	pop5	1.58743
1	pop6	1.58743
2	pop7	2.40718
3	pop8	2.68043
5	pop9	5.37552

Figure 3.5: Dendrogram Based Nei's (1972) Genetic distance between the DNA samples of plantlets from MS media with different hormonal concentration and mother plant using UPGMA- Modified from NEIGHBOR procedure of PHYLIP Version 3.5. pop1-pop 8 are the DNA samples of regenerants from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J. curcas*

Table 3.8: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between the DNA samples of regenerated plantlets from MS media with different hormonal concentration and mother plant from RAPD profile. Pop 1-8 are the DNA samples of regenerants from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J. curcas*

pop ID	1	2	3	4	5	6	7	8	9
1	****	0.8125	0.7188	0.7188	0.6875	0.7188	0.6562	0.7188	0.6562
2	0.2076	****	0.9062	0.8438	0.7500	0.7188	0.7188	0.7812	0.7188
3	0.3302	0.0984	****	0.8125	0.7188	0.6875	0.7500	0.7500	0.8125
4	0.3302	0.1699	0.2076	****	0.9062	0.8750	0.8750	0.8750	0.8750
5	0.3747	0.2877	0.3302	0.0984	****	0.9688	0.9688	0.9688	0.9062
6	0.3302	0.3302	0.3747	0.1335	0.0317	****	0.9375	0.9375	0.8750
7	0.4212	0.3302	0.2877	0.1335	0.0317	0.0645	****	0.9375	0.9375
8	0.3302	0.2469	0.2877	0.1335	0.0317	0.0645	0.0645	****	0.8750
9	0.4212	0.3302	0.2076	0.1335	0.0984	0.1335	0.0645	0.1335	****

The dendrogram (Figure 3.5) created from the RAPD agarose profile shows a small variation in the regenerants from different hormonal combinations. The samples can be categorized into two groups according to their genetic similarities. The samples S4- S8 and S9 encompass in one group. Pairwise similarity computed for each method separately is given in Table: 3.12. Among the plantlets from different samples, the highest similarity (0.96) was between 0.4 mg/L IBA and 2.0 mg/L BAP (S5) and 0.4 mg/L IBA and 2.5 mg/L BAP (S6). The highest similarity with mother plant (0.93) showed by the regenerants from hormonal combination 0.4 mg/L IBA and 4.0 mg/L BAP (S7) where S1 (plantlet from 2.0 mg/L BAP) showed the least similarity (0.65) with mother plant.

III.3.3. EVALUATION OF VARIATION IN CALLUS CULTURES AND REGENERATED PLANTS OF *J. CURCAS* USING ISSR MARKERS

III.3.3.1. ISSR Marker analysis in callus cultures

Only 4 among 20 ISSR primers found to be giving reliable bands. There were 34 scorable bands generated by 4 primers (Table 3.9). The number of amplicons per primer ranges from 3 for ISSRu4 (Figure 3.6 d) and 12 for ISSR18 (Figure 3.6 c). The average number of bands per primer was 8.50. Out of 34 bands, 24 were polymorphic (70.58%). The average number of polymorphic ISSR bands was 6.0 per primer.

In the dendrogram (Figure 3.7) constructed from the ISSR banding pattern variation in the callus regenerated from different hormonal combinations, could be seen. The samples were grouped into two main divisions. The samples S6, S7, S8 and S9 comprises in one group as they have more genetic similarity. The second group formed by 3 subgroups, in which S2 and S3 created one group and S4 and S5 created another group. S1 is seemed to be

Another distinct group is formed by S2, S3 and S4. However S1 was found to be diverged from the two sub groups. Pairwise similarity computed for each method separately is given in Table: 3.10. Among the callus from different samples, the highest similarity (0.85) was between callus from hormonal combination 0.2 mg/L IBA and 2.0 mg/L BAP (S2) and 0.3 mg/L IBA and 2.0 mg/L BAP (S3), while the least similarity (0.52) was between the callus from MS supplemented with 0.2 mg/L IBA and 2.0 mg/L BAP (S2) and 0.6 mg/L IBA and 2.0 mg/L BAP (S8) (Table 3.6). Similarly, the highest similarity with mother plant (0.88) showed by the callus from hormonal combination 0.4 mg/L IBA and 2.5mg/L BAP (S6) where S1 (callus from 2.0 mg/L BAP) showed the least similarity (0.63) with mother plant.

Table 3. 9 Primers used for ISSR analysis and the band characteristics obtained for the callus from different hormonal combinations and mother plant

Primers	Sequences	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (P%)	Total bands amplified
ISSR 15	ACC ACC ACC ACC ACC ACC	10	7	3	70.0	71
ISSR 18	TGC TGC TGC TGC TGC TGC	12	7	5	58.3	87
ISSR 19	CTA GCT AGC TAG CTA G	9	7	2	77.7	38
ISSR u4	GAG AGA GA G AGA GAG AA	3	3	0	100	16

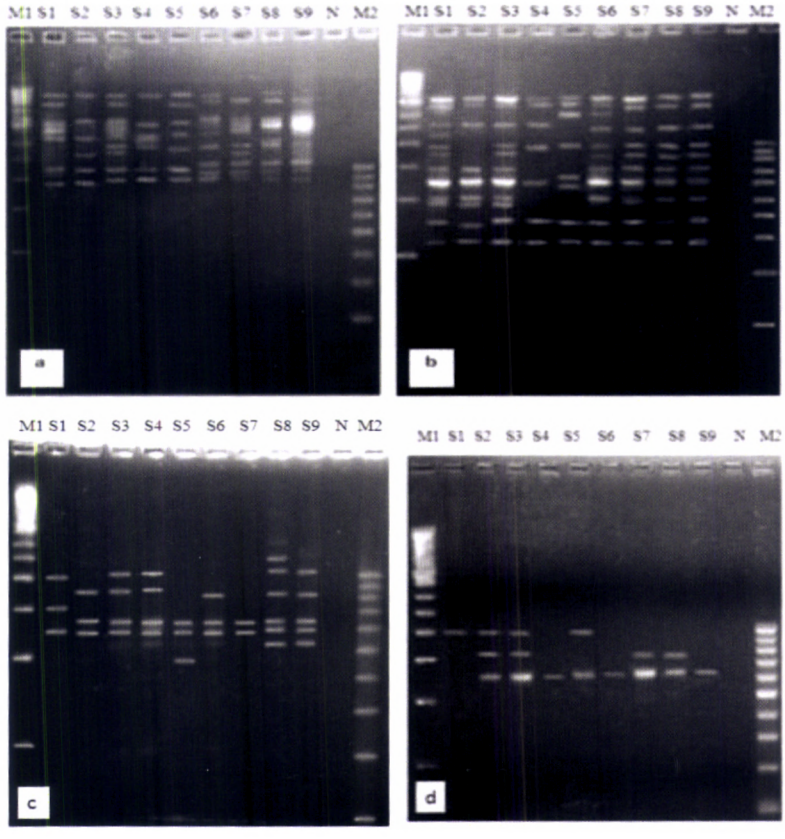


Figure 3.6: Agarose profile of ISSR amplicons generated by primer (a) ISSR-15, (b) ISSR- 18, (c) ISSR-19 and (d) ISSR- u4. Lane M1 –DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from callus from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J.curcas*; Lane N: Negative control; Lane M2 –DNA marker from Step Up Genei (100bp)

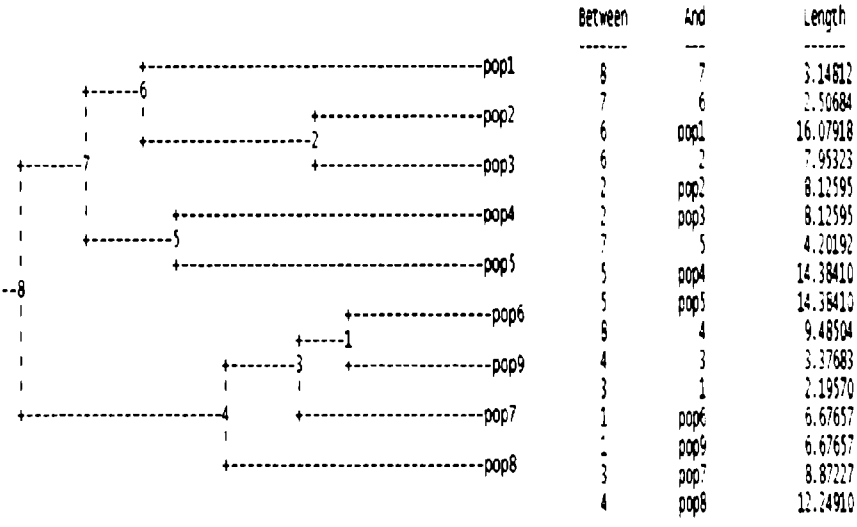


Figure 3.7: Dendrogram Based Nei's (1972) Genetic distance obtained by ISSR analysis between the DNA samples of callus from MS media with different hormonal concentration and mother plant using UPGMA- Modified from NEIGHBOR procedure of PHYLIP Version 3.5. S1-S8 are the DNA samples of calluses from MS media with different hormonal concentration, S9 Genomic DNA obtained from mother plant of *J. curcas*

Table 3.10: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) obtained from ISSR analysis between the DNA samples of callus from MS media with different hormonal concentration and mother plant from ISSR profile. pop 1-8 are the DNA samples of callus from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J.curcas*

pop ID	1	2	3	4	5	6	7	8	9
1	****	0.7250	0.7250	0.6750	0.6250	0.6000	0.6750	0.5500	0.6250
2	0.3216	****	0.8500	0.7000	0.6500	0.6750	0.6500	0.5250	0.6500
3	0.3216	0.1625	****	0.8000	0.7000	0.7250	0.6500	0.6250	0.7500
4	0.3930	0.3567	0.2231	****	0.7500	0.6750	0.6500	0.6250	0.7000
5	0.4700	0.4308	0.3567	0.2877	****	0.7250	0.7000	0.5750	0.6500
6	0.5108	0.3930	0.3216	0.3930	0.3216	****	0.8250	0.7500	0.8750
7	0.3930	0.4308	0.4308	0.4308	0.3567	0.1924	****	0.7750	0.8500
8	0.5978	0.6444	0.4700	0.4700	0.5534	0.2877	0.2549	****	0.8250
9	0.4700	0.4308	0.2877	0.3567	0.4308	0.1335	0.1625	0.1924	****

III.3.3.2. ISSR Marker analysis in regenerated plants

34 scorable bands were observed with the 4 primers used for genetic variation assessment in plant regenerated in different combinations (Table 3.11). The total number of amplicons produced per primer varied from 3 to 14 (Fig. 3.8). The average number of bands per primer was 8.5. 18 polymorphic bands could be seen in among total of 34 bands (52.9 %). The average number of polymorphic ISSR bands was 4.5 per primer.

Table 3. 11: Primers used for ISSR analysis and the band characteristics obtained for the regenerated plantlets from different hormonal combinations and mother plant

Primers	Sequences	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (P%)	Total bands amplified
ISSR 15	ACC ACC ACC ACC ACC ACC	12	6	6	50.0	94
ISSR 18	TGC TGC TGC TGC TGC TGC	14	5	9	35.7	113
ISSR 19	CTA GCT AGC TAG CTA G	5	4	1	80.0	34
ISSR u4	GAG AGA GA G AGA GAG AA	3	3	0	100.0	17

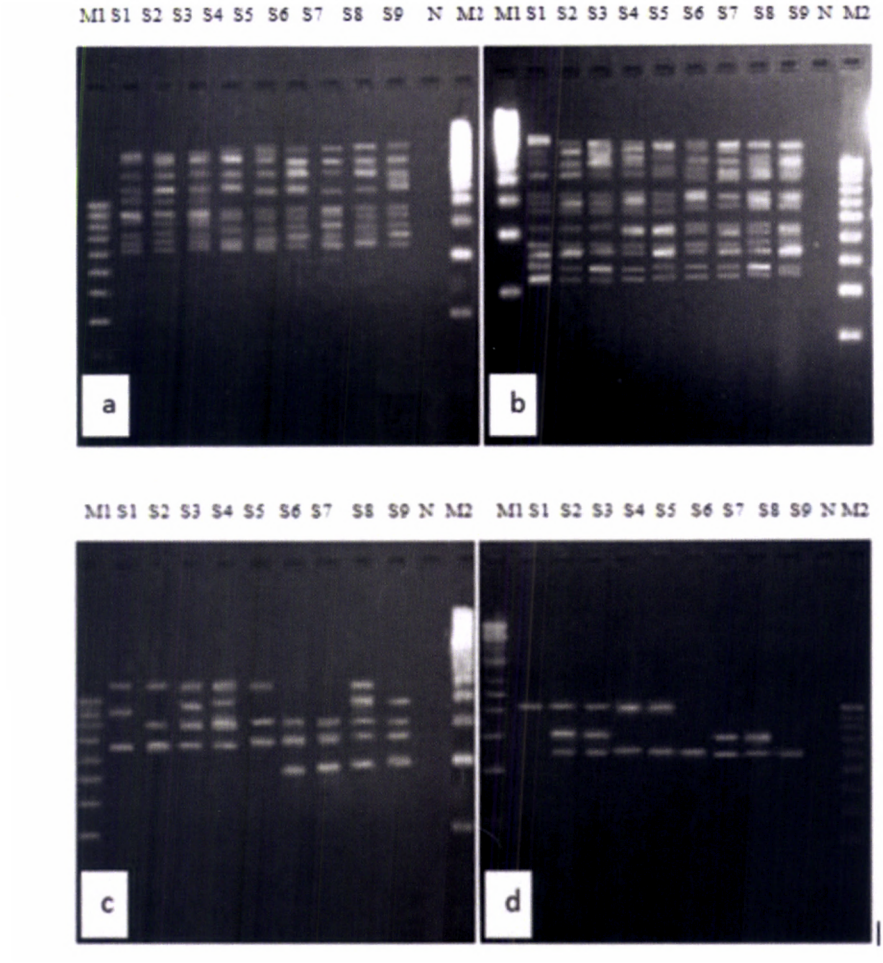
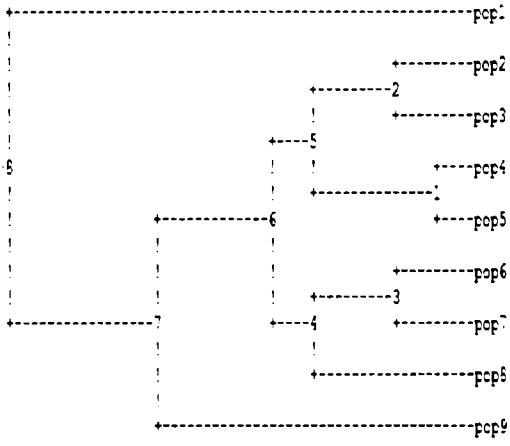


Figure 3.8: Agarose profile of ISSR amplicons generated by primer (a) ISSR-15, (b) ISSR- 18, (c) ISSR-19 and (d) ISSR- u4. Lane M1 –DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from regenerants from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J. curcas*; Lane N: Negative control; Lane M2 –DNA marker from Step Up Genei (100bp)



Between	And	Length
8	pop1	17.87979
8	7	5.66112
7	6	4.38187
6	5	1.56475
5	2	3.24082
2	pop2	3.03123
2	pop3	3.03123
5	1	4.77941
1	pop4	1.49265
1	pop5	1.49265
6	4	1.57864
4	3	3.22693
3	pop6	3.03123
3	pop7	3.03123
4	pop8	6.25816
7	pop9	12.21867

Figure 3.9: Dendrogram Based Nei's (1972) Genetic distance between the DNA samples of callus from MS media with different hormonal concentration and mother plant using UPGMA- Modified from NEIGHBOR procedure of PHYLIP Version 3.5. pop 1- pop 8 are the DNA samples of regenerants from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J.curcas*

Table 3.12: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) between the DNA samples of regenerants from MS media with different hormonal concentration and mother plant from ISSR profile. pop 1-8 are the DNA samples of regenerants from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J.curcas*

pop ID	1	2	3	4	5	6	7	8	9
1	****	0.7059	0.7647	0.7941	0.7647	0.6765	0.6176	0.6765	0.6176
2	0.3483	****	0.9412	0.8529	0.8824	0.8529	0.8529	0.7941	0.6765
3	0.2683	0.0606	****	0.9118	0.8824	0.8529	0.8529	0.8529	0.7353
4	0.2305	0.1591	0.0924	****	0.9706	0.8824	0.8235	0.8824	0.8235
5	0.2683	0.1252	0.1252	0.0299	****	0.9118	0.8529	0.8529	0.7941
6	0.3909	0.1591	0.1591	0.1252	0.0924	****	0.9412	0.8824	0.8235
7	0.4818	0.1591	0.1591	0.1942	0.1591	0.0606	****	0.8824	0.7647
8	0.3909	0.2305	0.1591	0.1252	0.1591	0.1252	0.1252	****	0.8824
9	0.4818	0.3909	0.3075	0.1942	0.2305	0.1942	0.2683	0.1252	****

From the dendrogram (Fig. 3.9) constructed, it is found that the samples S2-S8 and S9 (motherplant) are sharing high genetic similarity. S1 seemed to be distantly categorized indicating it is variation from the rest. Pairwise similarity computed for each method separately is given in Table: 3.12. Genetic identity among the samples ranges from 0.61 to 0.97, which says the samples have less genetic variation. Among the samples, the highest similarity (0.97) was between regenerant from hormonal combination 0.3 mg/L IBA and 2.5 mg/L BAP (S4) and 0.4 mg/L IBA and 2.0 mg/L BAP (S5), while the least similarity was between the plantlets from MS supplemented with 0.4 mg/L IBA and 4.0 mg/L BAP (S7) and 2.0 mg/L BAP (S1) (Table 3. 12). Similarly, the highest similarity with mother plant (0.88) showed by the plant from hormonal combination 0.6 mg/L IBA and 2.0 mg/L BAP (S8).

III.3.4. EVALUATION OF VARIATION IN CALLUS AND *IN VITRO* REGENERATED PLANTS OF *J. CURCAS* USING DAMD MARKERS

III. 3.4. 1. DAMD Marker analysis for callus

Consistent bands were given by 3 (HVA, M13 and 33.6) out of 5 primers used for DAMD analysis for callus genetic variation studies. There were 24 scorable bands generated by 3primers (Table 3.13). M13 and 33.6 have given 7 amplicons and HVA has given 10 amplicons upon PCR (Figure 3.10). The average number of bands per primer was 8.0. Out of 24 bands, 20 were polymorphic (83.3%). The average number of polymorphic DAMD bands was 6.66 per primer.

Table 3. 13: Primers used for DAMD analysis and the band characteristics obtained for the callus from different hormonal combinations and mother plant

Primers	Sequences	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (P%)	Total bands amplified
HVA	AGGATGGAAAGGAGGC	10	8	2	80.0	51
M13	GAGGGTGGCGGTTCT	7	6	1	85.71	37
33.6	AGGGCTGGAGG	7	6	1	85.71	29

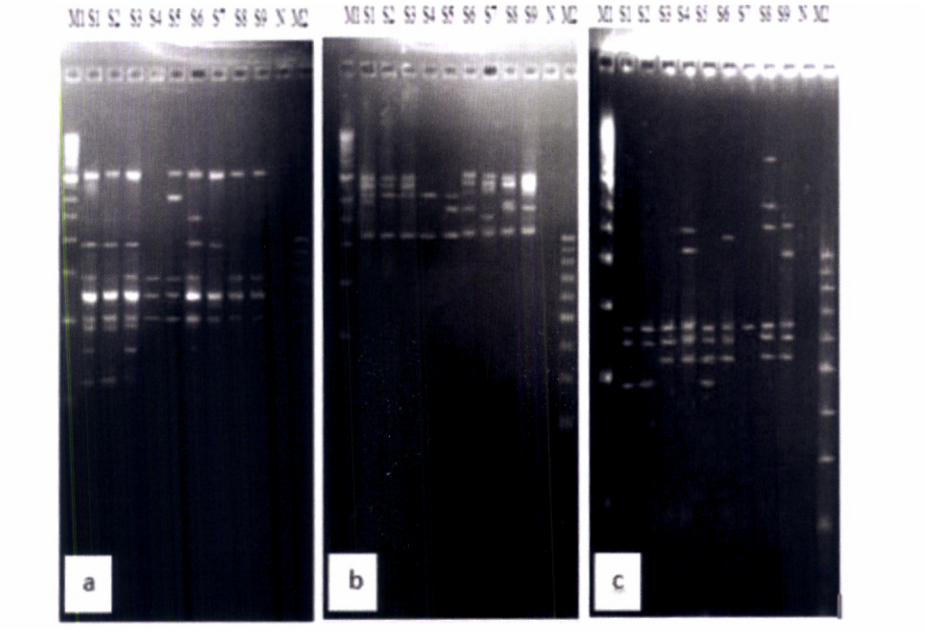
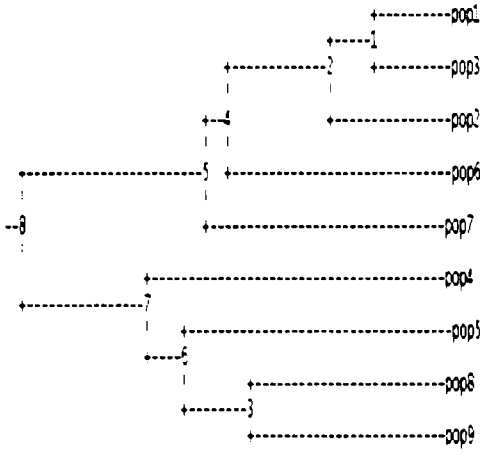


Figure 3.10: DAMD pattern generated by primer (a) HVA, (b) M13 and (c) 33.6. Lane M1 –DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from calluses from MS media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J.curcas*; Lane N: negative control; Lane M2 –DNA marker from Step Up Genei (100bp)



Between	And	Length
8	5	12.80651
5	4	0.86897
4	2	6.99922
2	1	3.12602
1	pop1	5.72052
1	pop3	5.72052
2	pop2	8.84654
4	pop6	15.84575
5	pop7	16.71472
8	7	8.50274
7	pop4	21.01849
7	6	2.39690
6	pop5	18.62159
6	3	4.68592
3	pop8	13.93567
3	pop9	13.93567

Figure 3.11: Dendrogram Based Nei's (1972) Genetic distance obtained by DAMD analysis between the DNA samples of callus from MS media with different hormonal concentration and mother plant using UPGMA- Modified from NEIGHBOR procedure of PHYLIP Version 3.5. pop 1- pop 8 are the DNA samples of callus from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J.curcas*

Table 3.14: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) obtained from DAMD analysis between the DNA samples of callus from MS media with different hormonal concentration and mother plant from DAMD profile. pop 1-8 are the DNA samples of callus from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J.curcas*

pop ID	1	2	3	4	5	6	7	8	9
1	****	0.8378	0.8919	0.5676	0.4324	0.7838	0.7568	0.5676	0.5946
2	0.1769	****	0.8378	0.6216	0.4324	0.6757	0.7027	0.6216	0.5946
3	0.1144	0.1769	****	0.6757	0.4865	0.7297	0.7027	0.6216	0.6486
4	0.5664	0.4754	0.3920	****	0.6486	0.4595	0.5405	0.6216	0.7027
5	0.8383	0.8383	0.7205	0.4329	****	0.4324	0.5135	0.7027	0.6757
6	0.2436	0.3920	0.3151	0.7777	0.8383	****	0.7027	0.6216	0.6486
7	0.2787	0.3528	0.3528	0.6152	0.6665	0.3528	****	0.5405	0.5676
8	0.5664	0.4754	0.4754	0.4754	0.3528	0.4754	0.6152	****	0.7568
9	0.5199	0.5199	0.4329	0.3528	0.3920	0.4329	0.5664	0.2787	****

The dendrogram (Figure 3.11) created from the DAMD agarose profile shows variation in the callus regenerated from different hormonal combinations. The samples can be categorized into two groups according to their genetic similarities. The samples S4, S5, S8 and S9 encompass in one group. The second group consists of S1, S2, S3, S6 and S7, in which S6 and S7 shows more identity than rest. Pairwise similarity computed for each method separately is given in Table: 3.14. Among the callus from different samples, the highest similarity (0.89) was between callus from hormonal combination 0.2 mg/L IBA and 2.0 mg/L BAP (S2) and 0.3 mg/L IBA and 2.0 mg/L BAP (S3). The highest similarity with mother plant (0.76) showed by the callus from hormonal combination 0.6mg/L IBA and 2.0 mg/L BAP (S8) where S1 (callus from 2.0 mg/L BAP) and S2 (callus from 0.2 mg/L IBA and 2.0 mg/L BAP) showed the least similarity (0.59) with mother plant.

III. 3.4. 2. DAMD Marker analysis for regenerated plants

There were 24 scorable bands generated by 3 DAMD primers (Table 3.9). The average number of bands per primer was 8.0. Out of 24 bands, 14 were polymorphic (58.3%). The average number of polymorphic DAMD bands was 4.66 per primer. DAMD primers HVA and M13 produced 4 and 3 monomorphic bands respectively in all samples (S1-S8 and in mother plant genomic DNA).

Table 3. 15: Primers used for DAMD analysis and the band characteristics obtained for the regenerant from different hormonal combinations and mother plant

Primers	Sequences	No. of Total bands	No. of polymorphic bands	No. of monomorphic bands	Percentage of polymorphic bands (P%)	Total bands amplified
HVA	AGGATGGAAA GGAGGC	10	6	4	60.0	66
M13	GAGGGTGGCG GTTCT	7	4	3	57.1	40
33.6	AGGGCTGGAG G	7	4	0	100.0	28

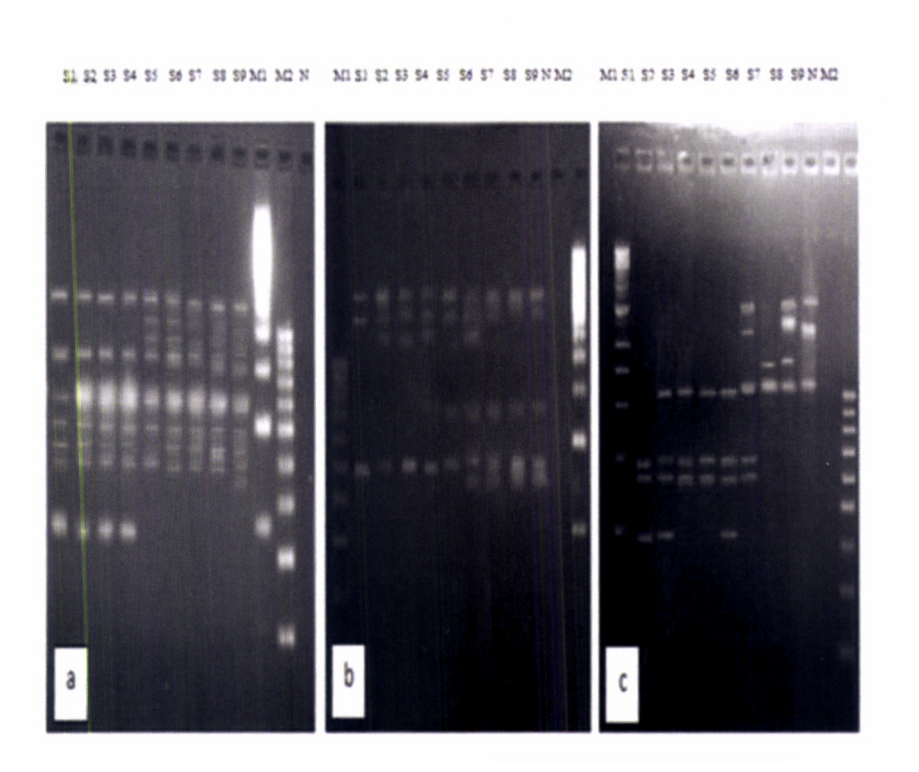
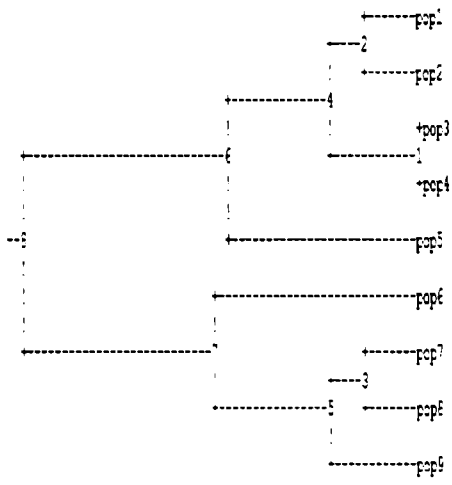


Figure 3.12: Agarose profile of DAMD amplicons generated by primer (a) HVA, (b) M13 and (c) 33.6. Lane M1 DNA marker from Chromous Biotech (250 bp); Lanes S1 – S8: Amplicons of genomic DNA obtained from regenerants from M.S media with different hormonal combination; Lane S9: Amplicons of genomic DNA obtained from mother plant of *J.curcas*; Lane N: Negative control; Lane M2 –DNA marker from Step Up Genei (100bp)



Between	And	Length
8	6	15.66992
6	4	7.65078
4	2	2.38275
2	pop1	4.35057
2	pop2	4.35057
4	1	6.73332
1	pop3	0.00000
1	pop4	0.00000
6	pop5	14.38410
8	7	14.66576
7	pop6	15.38826
7	5	8.65494
5	3	2.38275
3	pop7	4.35057
3	pop8	4.35057
5	pop9	6.73332

Figure 3.13: Dendrogram Based Nei's (1972) Genetic distance obtained by DAMD analysis between the DNA samples of regenerants from MS media with different hormonal concentration and mother plant using UPGMA- Modified from NEIGHBOR procedure of PHYLIP Version 3.5. pop 1- pop 8 are the DNA samples of regenerants from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J. curcas*

Table 3.15: Nei's genetic identity (above diagonal) and genetic distance (below diagonal) obtained from DAMD analysis between the DNA samples of regenerants from MS media with different hormonal concentration and mother plant from DAMD profile. pop 1-8 are the DNA samples of regenerants from MS media with different hormonal concentration, pop 9 represents genomic DNA obtained from mother plant of *J. curcas*

pop ID	1	2	3	4	5	6	7	8	9
1	****	0.9167	0.8333	0.8333	0.7500	0.5833	0.5417	0.4583	0.4583
2	0.0870	****	0.9167	0.9167	0.7500	0.6667	0.5417	0.4583	0.4583
3	0.1823	0.0870	****	1.0000	0.7500	0.6667	0.5417	0.4583	0.4583
4	0.1823	0.0870	0.0000	****	0.7500	0.6667	0.5417	0.4583	0.4583
5	0.2877	0.2877	0.2877	0.2877	****	0.7500	0.7083	0.6250	0.6250
6	0.5390	0.4055	0.4055	0.4055	0.2877	****	0.7083	0.7917	0.7083
7	0.6131	0.6131	0.6131	0.6131	0.3448	0.3448	****	0.9167	0.8333
8	0.7802	0.7802	0.7802	0.7802	0.4700	0.2336	0.0870	****	0.9167
9	0.7802	0.7802	0.7802	0.7802	0.4700	0.3448	0.1823	0.0870	****

The dendrogram (Figure 3.13) created from the DAMD agarose profile shows variation in the regenerants from different hormonal combinations. The samples S1-S5 formed one group, and S6- S9 covered in another group. Pairwise similarity computed for each method separately is given in Table: 3.15. Among the plantlets from different samples, the highest similarity (0.91) was between S1 (2.0 mg/L BAP), S2 (0.2 mg/L IBA and 2.0 mg/L BAP), S3 (0.3 mg/L IBA and 2.0 mg/L BAP) and S4 (0.3 mg/L IBA and 2.5 mg/L BAP). The highest similarity with mother plant (0.91) was shown by the regenerants from hormonal combination 0.6 mg/L IBA and 2.0 mg/L BAP (S8)

III.3.5. ANALYSIS OF SIGNIFICANCE OF VARIATION IN CALLUS AND REGENERANTS

In this study, total percentage of the polymorphism obtained in callus using RAPD, ISSR and DAMD was 90.24%, 75.6% and 58.3% respectively. 40.62%, 52.9 % and 58.3% polymorphism could be observed in regenerant using RAPD, ISSR and DAMD respectively. The results from the genetic identity analysis of callus and regenerants from different hormonal combinations using molecular markers, RAPD, ISSR and DAMD subjected to ANOVA and t test, to find out the significance. There was, no significant changes ($p>0.05$) in genetic variation index between callus and between regenerant from various hormonal combinations found. The combination 0.3 mg/L IBA and 2.5 mg/L BAP treated callus showed 0.43 ±0.13 variation. The variation that could be induced by 0.4 mg/L IBA and 2.0 mg/L BAP is 0.40±0.03, whereas the combinations, 0.3 mg/L IBA and 1.0 mg/L BAP contributed 0.38±0.08 variation. Results showed that 0.4 mg/L IBA and 4.0 mg/L BAP (0.31± 0.23) and 0.4 mg/L IBA and 2.5 mg/L BAP (0.28 ± 0.15) and 0.6 mg/L IBA and 2.0 mg/L BAP (0.38 ± 0.13) could induce lower level of variation than the rest.

Highest variation, 0.56± 0.19 was observed in 2.0 mg/L BAP. Hormonal combination 0.2 mg/L IBA and 2.0 mg/L BAP could induce variation 0.50 ±0.24, in plantlet stage, when detected by molecular markers whereas the combinations, 0.3 mg/L IBA and 1.0 mg/L BAP contributed 0.43 ±0.30 variation. The combination 0.3 mg/L IBA and 2.5 mg/L BAP treated plantlet showed 0.37 ±0.35variation. The variation that could be induced by 0.4 mg/L IBA and 2.0 mg/L BAP is 0.27 ±0.18. Results showed that 0.4 mg/L IBA and 2.5 mg/L BAP (0.22 ± 0.11) 0.4 mg/L IBA and 4.0 mg/L BAP (0.17± 0.10) and 0.6 mg/L IBA and 2.0 mg/L BAP (0.11± 0.02) could induce lower level of variation than the rest.

The amount of variation found in callus stage is lower in the treatments 0.2 mg/L IBA and 2.0 mg/L BAP, 0.3 mg/L IBA and 2.5 mg/L BAP, 0.4 mg/L IBA and 2.0 mg/L BAP, 0.4 mg/L IBA and 2.5 mg/L BAP, 0.4 mg/L IBA and 4.0 mg/L BAP and 0.6 mg/L IBA and 2.0 mg/L BAP in regenerated stage. However variation in plantlets was found to be more in the treatments 2.0 mg/L BAP and 0.3 mg/L IBA and 1.0 mg/L BAP. In total, variation in the regenerants (0.33± 0.24) was lower than in the callus stage (0.38 ±0.13).

Somaclonal variation is known to arise and cause genetic variability during the *in vitro* culturing of plant cells and tissues. Phytohormone treatments were effective in inducing genetic variability in *Saccharum officinarum* (Khan *et al.*, 2008). Plants regenerated from leaf base callus have shown variation at the DNA level during *in vitro* culture of *Curcuma longa* (Salvi *et al.*, 2001; Tyagi *et al.*, 2007). Somaclonal variation reduces the commercial value of plants (Oropeza *et al.*, 1995). Thus it is important to detect somaclonal variation at an early stage of plant growth to avoid economic loss (Chuang *et al.*, 2009). Cytological assessment has been proposed but not often used as it can be difficult in many species where chromosomes are difficult to observe. Analyses of secondary metabolites and isozyme patterns have also been used, but they are limited in their sensitivity (Morell *et al.*, 1995). One acceptable approach is to examine the genome of regenerants with the use of molecular markers (Bogani and Simoni, 2001). Molecular markers are being increasingly utilized as a useful marker reflecting changes in genetic level associated with growth or stress. Sequence variation arising through the culture process has been detected in several different ways (Kaeppler *et al.*, 1998; Olhoft and Phillips, 1999) including random genome scanning using RFLPs or RAPDs, and analysis of specific mutants.

III.4.1. Hormonal supplementation with 0.2 mg/L IBA and 2.0 mg/L BAP could induce highest variation in callus and 2.0 mg/L BAP in regenerated plantlets

The current study showed the influence of supplementation of MS media with different combinations of auxin (IBA) and cytokinin (BAP) on sequence changes in the DNA extracted from callus and regenerated plants. Hormonal combination 0.2

mg/L IBA and 2.0 mg/L BAP could induce highest variation in callus. However plants regenerated from callus grown in 2.0 mg/L BAP treatment was found to show maximum variation in plantlet stage. It is possible that the hormonal treatments used in tissue culture which induce a high growth rate may cause variations in DNA sequences (Bairu *et al.*, 2006).

In a study by Sonia *et al.* (2001) genetic variation and high polymorphism could be detected in the regenerants from callus grown in MS medium with IBA. Narayanaswamy (1977) reported that the toxicity caused by an excess of growth regulators in the culture medium, or the extended period of time in which the culture was exposed to them, might lead to genetic, physiological and morphological changes, resulting in a reduction of the proliferation rate *in vitro*. It is possible sometime that growth regulators act as mutagens. It was reported that lower level of auxin greatly reduced variability in regenerated *Hordeum* plants, compared with a high concentrations (Deambrogio and Dale, 1980). Fewer variants were produced more often on media containing low concentrations of NAA or IBA (Ahmed *et al.*, 2003). Contradictory to this, our study showed that low concentration of auxin contributes to DNA sequence variation. And in the absence of auxin, change in the morphology of the callus was evident. Thus in our study changes in auxin (IBA) level seemed to affect genetic fidelity in the callus formed. Auxin was found to induce somaclonal variation in *Solanum melongena* by Hitomi *et al.* (1998) and observed as NAA induced variation higher than 2,4-D. It has been reported that 2,4-D did not have a direct mutagenic effect but caused abnormalities indirectly by stimulating disorganized growth (Bayliss, 1980). According to Saieed *et al.* (1994) this variation may be a consequence of the stress inherent in cellular deprogramming induced by plant growth regulators. Epigenetic changes resulting in altered hormone response have been reported for several species (Meins, 1989). In the present study also it was

observed that slight variation in the hormonal concentration caused DNA sequence changes in the cells of callus and regenerated plants.

One of the possible explanations for the polymorphism could be a different copy number of the corresponding DNA loci in the samples under study (Stegni *et al.*, 2000). Lei *et al.* (2006) in his study in oil palm reported that the tissue culture process induces changes in methylation in regenerants at many sites within the genome, although the possibility that certain sequences are more predisposed than others to alterations of this kind cannot be excluded. Polymorphism in regenerants of Peach (*Prunus persica*) could be revealed by RAPD markers (Hashmi *et al.*, 1997). Qualitative mutation is frequent among tissue culture regenerants and the summation of protein assays, random DNA studies, and specific mutant analyses suggests that single-base changes, or very small insertions/deletions, are the basis of these changes (Kaeppler, 1998). The culture environment especially the choice and the concentration of growth regulators in the medium influences somaclonal variation (Karp, 1982).

Matzke and Matzke (1996) provide evidence that pre-existing variation in the explant should not be overlooked, especially in the case of epimutations. Somaclonal variations are mainly caused by newly generated mutations arising from tissue culture process (Sato *et al.*, 2011). If epigenetic patterns are part of the normal pattern of development, then certain explants which have reached an advanced stage of development produce callus representing cells with different epigenetic patterns (Shawn *et al.*, 2000). Studies of qualitative mutations from tissue culture indicate that mutations accumulate sequentially with time in culture (Fukui, 1983; Zehr *et al.*, 1987).

The deviation from the normal developmental pattern may be important for totipotency, but may also cause phenotypic variation which is not meiotically

heritable (Shawn *et al.*, 2000). Brettel *et al.* (1986) and Dennis *et al.* (1987) elucidated instances in which a mutant phenotype has occurred by two independent mutations at the Adh1 locus and is a result of A-to-T transversion mutations. Transposons and retrotransposons are activated by the culture process (Peschke *et al.*, 1987; Peschke *et al.*, 1991; Brettell and Dennis, 1991; Hirochika *et al.*, 1996). Transposable element activation has been shown to be induced by genomic shock (McClintock, 1984).

Phillips *et al.* (1994) suggested that a repeat induced-point mutation (RIP) or methylation induced premeiotically (MIP) type mechanism could explain both the high frequency of base change and of methylation change. This hypothesis suggests that duplicate sequences in the genome, which peacefully coexisted under normal cellular conditions, begin to interact under the stress conditions of culture inducing a mutagenic process. As pointed out by Matzke and Matzke (1996) even if mutation is not occurring by a Repeat Induced Point mutation process, it is still conceivable that an increased frequency of deamination of methylated cytosine or its metabolic precursors may raise the mutation rate indirectly through a methylation-based mechanism.

III.4.2. Hormonal combination 0.6 mg/L IBA AND 2.0 mg/L BAP induces lower genetic variation in callus and regenerants

The availability of growth hormones in the culture media regulates the genetic pattern. Molecular markers can be used to detect somaclonal variation in order to identify hormonal combinations and conditions to minimize the incorporation of genetic changes and to regenerate true to type plants. Low levels of DNA sequence variations indicate the genetic uniformity of the callus and regenerants in the media 0.6 mg/L IBA and 2.0 mg/L BAP, thereby establishing the suitability of this media for *in vitro* micropropagation of *J. curcas*. In a study by Razak *et al.* (2012), *in vitro*

clonal propagation using BAP and genetic fidelity analysis of the regenerants of *Spilanthes calva* using RAPD and ISSR marker, the banding profiles from micropropagated plants were similar to those of mother plant and genetic uniformity of *in vitro* clones confirmed by the dendrogram generated through UPGMA analysis in which 98 % similarity has revealed amongst them. Maharana *et al.*, (2012) reported genetic fidelity of regenerant from *J. curcas* nodal explant grown in IBA, BAP, AS, glutamine and proline using RAPD markers.

Variation can occur due to endogenous stimulus exerted by exogenous hormonal combination. Therefore in order to ensure the genetic stability of cultures, the exogenous auxin cytokinin stimulus provided by the culture maintenance medium should be studied and the concentration standardized for each species. Permanence of nuclear genome helps to maintain the genetic fidelity of the plant and its proper functioning at the cellular level. Genetic as well as biochemical stability of a population derived from tissue culture is important especially in transformation studies. Therefore the lack of variation reported in the regenerant is significant for biotechnological manipulations. Early detection of genetic variation is considered to be useful in plant tissue culture and transformation studies (Soniya *et al.*, 2001).

III.4.3. DNA sequence changes in callus did not reflect that in regenerated plants

In the current study, the DNA sequence variation which was detected in callus formed in different hormonal combinations was not reflected in the regenerated plants. This may be because; the highly altered callus cells would be unable to give regenerated plant. Study in tissue-cultured tomato plants by Soniya *et al.* (2001) indicated induction of random changes in the genomic organization during differentiation of tomato plants from callus culture under *in vitro* conditions. Callus-

derived plants of *Arabidopsis thaliana* carried a high frequency of point mutation and changes in the number of chromosomes (Gaj and Maluszynski, 1987).

III.4.4. DNA sequence changes in the regenerated plants was found to be lower than in the callus

Molecular markers can be used to detect somaclonal variation in order to identify hormonal combinations and conditions to minimize the incorporation of genetic changes and to regenerate true to type plants. In the present study, the variation detected by RAPD, ISSR and DAMD analysis was found to be lower in the regenerants than in the callus. This may be due to the heterogenous nature of the callus induced by hormonal and environmental stress under *in vitro* condition. Infidelity could result from physiological conditions, disruption in a methyl-directed mismatch repair system, or some other type of cellular stress (Shawn *et al.*, 2000). Somaclonal variation might be unstable or reversible, although certain epigenetic systems outside of tissue culture are quite stable for many generations (Patterson *et al.*, 1993; Cubas *et al.*, 1999).

Larkin *et al.* (1985) evidenced that transposable elements play a vital role on somaclonal variation. Siddiqui *et al.* (1994) opinioned that one did not know the phase at which the variation arise. According to them the variations are caused by a combination of physical and chemical phenomenon.

III.4.5. DAMD analysis was found to be superior to RAPD and ISSR analysis in the detection of sequence changes induced in callus and regenerated plants

Molecular marker study was effective in detecting variations that may occur in callus induced from leaf explants of *J. curcas*. Molecular methods like RAPD, RFLP, ISSR and DAMD methods are preferable for detecting unidentified or

unspecified mutations and determining whether a mutation has occurred (Sato *et al.*, 2011). The comparative analysis of the three markers (RAPD, DAMD and ISSR) showed that DAMD is more powerful than RAPD and ISSR in assessment of genetic diversity in pomegranates (Kanwar *et al.*, 2010). In the current study also DAMD analysis was found to be more effective in the detection of sequence changes in the callus as well as regenerated plants among the three markers used, RAPD, ISSR and DAMD. Since minisatellite core sequences which are used as primers are longer than RAPD-PCR primers, DAMD-PCR can be effectively carried out at relatively high stringency reactions (Mehmet and Ayse, 2008). In comparison to RAPD markers, which are one of the most commonly used marker types, DAMD-PCR markers are more reproducible and reliable due to the effective amplification carried out at relatively high PCR stringencies (Mihalte *et al.*, 2011).

III.4.6. Mitotic index and percentage of shoot response decreases with increase in variation index in callus

Mitotic index and percentage of shoot response was found to decrease with increase in variation index in callus. The study is important because the variation induced during *in vitro* culture condition may affect the regenerants of the same. These changes may be due to the sequence variations in loci revealed by the primers used in the donor plant DNA or may be due to sequences outside these loci which results in new amplicons appearing in addition to the majority of fragments characteristic of donor plant DNA. Soh and Yang (1993) studied the effects of plant growth regulators on mitotic activity and the chromosomal behavior and concluded that they increased the mitotic activity at lower concentration but the cytokinins kinetin and BAP were not as effective as auxins to increase the mitotic index. According to Mendoza and Kaeppler (2002), the use of auxin in combination with

cytokinin, leads to rapid cell division, forming a large number of relatively small and undifferentiated cells. Cytokinins may elevate cell division rates by induction of expression of CycD3, which encodes a D-type cyclin thought to play a role in the G1-M transition of the cell cycle. However the role of cytokinins in other aspects of the cell cycle remains unclear (D'Agostino and Kieber, 1999). The production of ATP is of great importance during cell division as it is needed to synthesize the different enzymes and provide energy needed for cell division (Klug *et al.*, 2006).

Plant growth substances (auxins, cytokinins, gibberellins, abscisic acid, ethylene) and some putative regulatory substances (jasmonates, brassinosteroids, polyamines) are shown to control (modulate) gene expression in developmental systems in interacting ways (Parthier, 1989). A remarkable feature about the mode of action of auxin is that it induces changes in global gene expression within 5 to 15 min of application (Abel *et al.*, 1994); this remains one of the fastest regulations of gene transcription known. Changes in protein level are detectable around 15 to 30 min after auxin application (Oeller and Theologis, 1995). It has been proposed that sustained growth involves both gene expression and stimulation of growth-limiting processes (Vanderhoef *et al.*, 1976).

Somaclonal variability often arises in tissue culture as a manifestation of epigenetic influence or changes in the genome of differentiating vegetative cells induced by tissue culture conditions (Larkin and Scowcroft, 1981; Muller *et al.*, 1990). So, the negative correlation of genetic variation induced in different combination with mitotic index and shoot induction in the current study indicates that the variation in hormonal combination can bring out genetic changes and thereby the changes in the cell division pattern and growth.

CHAPTER IV: PHYTOCHEMICAL STUDIES IN CALLUS, REGENERANTS AND MOTHER PLANT

IV.1. REVIEW OF LITERATURE

IV.1.1. SECONDARY METABOLITES IN *JATROPHA CURCAS*

Phytochemical analysis of methanolic extracts of root, stem and petiole of *J. curcas* showed the presence of alkaloid, saponins, tannins, terpenoids, steroids, glycosides, phenols and flavonoids (Sharma *et al.*, 2012). Maximum phenolic content was found in leaf extract and flavonoid content in latex of the plant (Sharma *et al.*, 2012). Alkaloids, saponins, tannins, anthraquinones and flavonoids are known to have activity against several pathogens and suggest their traditional use for the treatment of various illnesses (Hassan *et al.*, 2004; Usman *et al.*, 2007). *J. curcas* has shown to possess insecticidal as well as antimicrobial properties (Adebowale and Sdedire, 2006; Adamu *et al.*, 2006; Kisangu *et al.*, 2007).

Terpenoid compounds are the major secondary metabolites found in the *Euphorbiaceae* family. Among terpenes, diterpenoids have dominated research in Jatropha species with respect to their novel chemical structures and medicinal values (Devappa *et al.*, 2011). The antimicrobial activity was found in different *J. curcas* parts like leaves, stem bark, root bark, root wood and kernel seeds (Namuli *et al.*, 2011).

IV.1.1.1. LEAF EXTRACT

Ethyl acetate extracts of leaves of *J. curcas* contain a complex of 5-hydroxypyrrolidin-2-one and pyrimidine-2, 4-dione (uracil) (Staubmann *et al.*, 1999). From the latex of *J. curcas*, a novel cyclic octapeptide was isolated and named curcacycline A (Berg *et al.*, 1995). Diterpene compounds such as tigliane (Guette *et al.*, 1999), jatrophone (Marquez *et al.*, 2005) and dinorditerpene (Naengchomnong, 1986) and a triterpenoid, 3-O-acetylaleuritolate acid (Ling *et*

al., 1996) have been identified from this plant (Marquez *et al.*, 2005). Aqueous extracts of leaves of *J. curcas* were evaluated for antihelmintic activity on adult Indian earthworms *Pheretima posthuma* (Ahirrao *et al.*, 2009). Methanolic extract of *J. curcas* plant has shown antiulcer activity on aspirin-induced gastric lesions in Wistar rats (Kannappan *et al.*, 2008). The juice of the leaves has both procoagulant and anticoagulant activities (Osoniyi and Onajobi, 2003). The antimicrobial and larvicidal activities of the plant leaves have been reported by Kalimuthu *et al.* (2010; 2011).

Zeng *et al.* (2004) studied the *in vitro* antibiotic effect of alcohol extract from Jatropha leaf on *Escherichia coli* and *Staphylococcus aureus*. The extract inhibited *E. coli* and *S. aureus*, and the activity against *E. coli* was found to be better than that against *S. aureus*. Sanis *et al.* (2012) reported the effect of ethanolic extract of leaves of *J. curcas* for developing a safe and ecofriendly therapeutic agent to combat the problems of tick and tick-borne diseases. The extract caused significant blocking of hatching of the laid ova in the treated ticks. In a study by Yusuf and Maxwell (2011) using hot plate and tail flick mice models, the oral administration of *J. curcas* extract at the doses of 100, 200 and 400 mg/kg showed potent analgesic effect. The extracts also have been shown to have a potent cardiovascular action (Fojas *et al.*, 1986). Ritwik *et al.* (2012) in their study found that *J. curcas* leaf extracts showed effective anti-viral and probable entry inhibition activity against potentially drug-resistant HIV.

IV.1.1.2. ROOT EXTRACTS

The root of *J. curcas* is a rich source of diterpenes with daphnane and lathyrane skeletons (Naengchomnong, 1986). Antibacterial, antifungal activities of methanolic extracts of root of *J. curcas* have been reported by Kumar *et al.* (2012). Recently, Aiyelaagbe *et al.* (2007) reported that the presence of steroids, alkaloids and saponins in the root extract of *J. curcas* inhibited *Candida albicans, Neisseria gonorrhoea* and *Staphylococcus aureus*. This may be attributed to the

presence of soluble phenolic and polyphenolic compounds (Kowalski and Kedzia, 2007).

IV.1.1.3. LATEX AND FRUITS

The latex of *Jatropha* contains an alkaloid known as "Jatrophine" which is believed to be anticarcinogenic (Thomas *et al.*, 2008, Lin *et al.*, 2003). It is also used externally for skin diseases and rheumatism and for sores on domestic livestock (Thomas *et al.*, 2008; Salas *et al.*, 1994) and the treatment of piles (Thomas *et al.*, 2008; Sarika and Meenakshi, 2008). The latex of *J. curcas* showed antibacterial activity against *Staphylococcus aureus* (Thomas, 1989). *J. curcas* latex and twigs have been used for cleaning teeth (Osoniyi and Onajobi, 2003). The fruits of *J. curcas* and the stem bark of *Cochlospermum planchonii* are combined for the treatment of diabetes mellitus (Igoli *et al.*, 2005). Li *et al.* (2004) studied the insecticidal activity of ethanol extract from Jatropha seed against *Lipaphis erysimi* (Kaltenbach). Use of aqueous extract of the seed and nut as contraceptive has been reported (Goonasekera *et al.*, 1995).

IV.1.1.4. STEM AND PETIOLE EXTRACTS

The *in vitro* antimicrobial activity of crude ethanolic, methanolic and water extracts of stem bark of *J. curcas* against *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Streptococcus faecalis, Staphylococcus epidermidis, Shigella dysentriae, Micrococcus kristinae, Klebsiella pneumoniae, Bacillus cereus, Bacillus subtilis, Proteus vulgaris* and *Serratia marcescens* were investigated (Igbinosa *et al.*, 2009). The sap from the stem is used to stop bleeding from wound (Gadekar, 2006). Methanolic extracts of stem and petiole of the plant showed, antibacterial, antifungal activities against gram-positive and gram negative bacteria with varying magnitudes (Kumar *et al.*, 2012).

IV.1.2. SOLVENT SYSTEM FOR EXTRACT PREPARATION

Plants have limitless ability to synthesize aromatic secondary metabolites, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963) that play vital role in medical industry. Extraction methods involve separation of medicinally active fractions of plant tissue from inactive/inert components by using selective solvents and extraction technology (Green, 2004). Since nearly all of the identified antimicrobial compounds from plants are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Serkedjieva and Manolova, 1992). Thus the most commonly used solvents for preliminary investigations of antimicrobial activity in plants are methanol, ethanol and water (Parekh *et al.*, 2006; Bisignino *et al.*, 1996; Lourens *et al.*, 2004; Salie *et al.*, 1996; Rojas *et al.*, 2006).

IV.1.3. VARIATION IN PHYTOCHEMICAL PRODUCTION THROUGH *IN VITRO* CULTURE

In the search for alternatives for production of desirable medicinal compounds from plants, biotechnological approaches, specifically, plant tissue cultures, are found to have potential as a supplement to traditional agriculture (Rao and Ravishankar, 2002). Some of the medicinal compounds localized in morphologically specialized tissues or organs of native plants have been produced in culture systems not only by inducing specific organized cultures, but also by undifferentiated cell cultures. The possible use of plant cell cultures for the specific biotransformations of natural compounds has been demonstrated (Cheetham, 1995; Scragg, 1997; Krings and Berger, 1998; Ravishankar and Rao, 2000). In order to obtain high yields suitable for commercial exploitation, efforts have focused on isolating the biosynthetic activities of cultured cells, achieved by optimizing the cultural conditions, selecting high-producing strains, and

employing precursor feeding, transformation methods, and immobilization techniques (Dicosmo and Misawa, 1995).

The production of high yield of secondary compounds has been reported from callus culture, from suspension culture or by using precursor (Cusido *et al.*, 1999, Croteau *et al.*, 2000, Jordan *et al.*, 2006). Advances in tissue culture, combined with improvement in genetic engineering, specifically transformation technology, have opened new avenues for high volume production of pharmaceuticals, nutraceuticals, and other beneficial substances (Hansen and Wright, 1999). Large scale plant tissue culture is found to be an attractive alternative approach to traditional methods of plantation as it offers a controlled supply of biochemicals independent of plant availability (Sajc *et al.*, 2000). In recent days, apart from other conservational measures, major efforts have been directed towards callus and cell suspension cultures for the production of secondary metabolites of pharmacological and pharmaceutical interests (Hedge *et al.*, 2010). Kieran *et al.* (1997) detailed the impact of specific engineering-related factors on cell suspension cultures. Current developments in tissue culture technology indicate that transcription factors are efficient new molecular tools for plant metabolic engineering to increase the production of valuable compounds (Gantet and Memelink, 2002).

If tissue-cultured superior plants can be cloned in large numbers, they can relieve pressure on plants in the wild. Gawri and Upadhyay (2012) observed that the secondary metabolites like alkaloids, glycosides, flavonoids, tannins and phlobatannis were present in the fresh samples as well as the cultured callus of *J. curcas*. But the concentration of alkaloids and glycosides were higher in the callus. Consequently, increased production of secondary metabolites can be obtained by tissue culture techniques. *In vitro* raised plants of *Salvia stenophylla* were higher in a-bisabolol content (Musarurwa *et al.*, 2010). Similarly, Devil's Claw tubers from culture derived stock had higher iridoid content than those of wild plants (Bairu *et al.*, 2011).

The content of the gastroprotective diterpene solidagenone, phenolics chlorogenic acid (CA) and rutin was studied in rhizomes from wild growing *Solidago chilensis* and in callus and in *in vitro* regenerated plantlets by analytical HPLC. In terms of dry starting material, the percentage of solidagenone content in nine *S. chilensis* samples ranged from 0.5- 3.5% for rhizomes from wild growing plants, 0.1-0.3% for callus and 0.3% for an *in vitro* regenerated plantlet, respectively (Guillermo *et al.*, 2005). When screened for presence of phytochemicals, alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, saponins and reducing sugars were found to be present in methanol, acetone, chloroform and water extracts of leaf and *in vitro* grown callus of *Centella asiatica* (Thangavel *et al.*, 2011).

In vitro cell cultures of two Papaver species, *Papaver somniferum* and *Papaver bracteatum* initiated from mature seeds were screened for their ability to produce alkaloids. Undifferentiated callus produced small amounts of sanguinarine, which increased with the degree of tissue differentiation. Thin layer chromatography analysis revealed variations in alkaloid spectrum between parallel cell lines (Alkhimova *et al.*, 2001).

IV.1.4. VARIATION IN ANTIBACTERIAL ACIVITY IN REGENERANTS

Kalimuthu *et al.* (2010) reported that methanol extract from leaf callus of *Jatropha curcas* at high concentrations (1.0 and 1.2%) inhibited the growth of *Staphylococcus aureus* and *Pseudomonas sp.* with diameter of inhibition 20 and 23 mm respectively. Though, the antifungal activities of leaf extract were noteworthy, the methanol extract of leaf derived callus of *J. curcas* showed higher antifungal activity with concomitant increase in concentrations. In a study by Gawri and Upadhyay (2012) petioles of fresh leaves of *J. curcas* were cultured in MS media and when explants and callus so obtained were tested for antimicrobial activity, both the samples showed antimicrobial activity.

In a study by Thenmozhi and Sivaraj (2011) callus was induced and antibacterial activity of Petunia leaf and callus extracts in different solvents like, petroleum ether, chloroform, ethyl acetate, ethanol and aqueous extract was studied against *Escherichia coli, Pseudomonas aeroginosa, Bacillus subtilis, Salmonella spp., Streptococcus spp.* and *Staphylococcus aureus.* The highest antibacterial activity was recorded in chloroform leaf extract against *Escherichia coli* (23±0.33 mm) and least activity in callus aqueous extract against *Pseudomonas aeroginosa* (6±0.42 mm).

When antimicrobial activity of crude chloroform, acetone, ethanol and water extracts from *Alopyllus cobbe* L. (Sapindaceae) leaf and leaf callus were tested in three different concentrations (250, 200 and 150 g/disk) against two gram positive and two gram negative strains *Bacillus subtilis, Streptococcus faecalis, Escherichia coli* and *Pseudomonas aeruginosa*, by Hedge *et al.* (2010), acetone extract showed maximum activity in both leaf and leaf callus extracts. The zone of inhibition was higher in callus extracts, when compared to that of leaf extracts. In an analysis on antibacterial activity of leaves and internodal callus extracts of *Mentha arvensis* L., bio-efficacy study confirmed that the calli mediated tissues showed the maximum zone of inhibition. (Johnson *et al.*, 2011).

Study in antibacterial activity of leaf and callus extracts of *Centella asiatica* by Thangavel *et al.* (2011) reported that all the extracts from leaf and callus of *C. asiatica* were showed significant antibacterial activities against the tested organisms. However, methanol extracts of leaf and callus showed maximum inhibitory effect against *Bacillus cereus, Escherichia coli, Staphylococcus aureus* and *Pseudomonas aeruginosa.* Antimicrobial activity in mother plant and callus extracts of *Solanum trilobatum* L. was studied against *Escherichia coli, Staphylococcus aureus, Aspergillus flavus* and *Aspergillus niger* by Nagarajan (2009) and stem and leaf callus extracts have been reported to be showed significant activity against the tested microorganisms than the mother plant.

The antibacterial efficacy of the wild aerial, inflorescence parts of *Artemisia amygdalina* was investigated by Rasool *et al.* (2012) and compared with tissue culture regenerants such as *in vitro* grown plant, callus, and green house acclimatized plant antibacterial activity. Methanolic and ethyl acetate extracts of both wild and regenerant plants were active against *Pseudomonas aeruginosa, Shigella flexneri, Acinetobacter* spp., *Listeria monocytogenes, Vibrio cholerae* and *Salmonella paratyphi.*

IV. 2. 1. COLLECTION OF PLANT MATERIAL

Analysis of variation in bioactive compounds of extracts from mother plant, *Jatropha curcas* L. (TBGT 70000), callus generated from leaf explant on 1.5 mg/L BAP and 0.4 mg/L IBA and plants regenerated from callus on media containing 0.2 mg/L BAP and 0.4 mg/L IBA was done. In the study, extracts taken from each plant part of four regenerants and compared with the mother plant. Fresh leaves, petioles, stem and root were harvested from regenerants and mother plants and washed with distilled water and tween 80 solution so as to remove dust and other foreign particles. The samples were then left on a clean surface and were air-dried in the shade for 10-12 days. The dried samples were pulverized to fine powder using an electric grinder and stored in air tight bottles. The powdered material was used, for preparation of extracts for studying the variation in *in vivo* and *in vitro* generated plants.

IV. 2. 2. PREPARATION OF EXTRACTS

0.25 g of the powdered plant material was cold extracted in analytical grade ethanol, methanol and distilled water. The extracts were then centrifuged for 5 minutes at 10,000 rpm (Sigma laboratory centrifuge 3K30; rotor: 12154). The supernatant was collected and observed for its physical properties. It was then concentrated to dryness by evaporation. The dried samples were re dissolved (10 mg/mL) in corresponding solvents.

IV. 2. 3. PHYTOCHEMICAL ANALYSIS OF PLANT EXTRACTS.

The following tests were done to detect the presence of active chemical constituents like alkaloids, tannins, glycosides, flavonoids, anthraquinones, oils and fats and saponins in callus extracts and in leaf, petiole, stem and root extracts.

IV. 2. 3. 1. Flavonoids

IV. 2. 3. 1.1. Sulphuric acid test

A few drops of 10% concentrated sulphuric acid was added to solvent free extract, followed by 1mL of ammonia. (Siddiqui and Ali, 1997).

IV. 2. 3. 1. 2. Shinoda test

To the test Solution, few fragments of Magnesium ribbon was added followed by drop wise addition of concentrated Hydrochloric acid.

IV. 2. 3. 2. Glycosides.

0.5g of each extract was dissolved in 2 mL of chloroform. H_2SO_4 was carefully added to form a lower layer.

IV. 2. 3. 3. Phenolics

About 2mL of the extract was measured into a test tube and three drops of ferric chloride solution added.

IV. 2. 3. 4. Saponins

About 2mL of the extract was measured into a test tube and shaken vigorously.

IV. 2. 3. 5. Tannins

1 mL of 5% ferric chloride to solvent free extract was added and observed for the formation of bluish black or greenish black precipitate (Siddiqui and Ali, 1997).

IV. 2. 3. 6. Sterols and Triterpenoids

IV. 2. 3. 6.1. Salkowski test

Treated extract in Chloroform with few drops of conc. Sulphuric acid, shaken well and allowed to stand for some time. Observed for red color appears at the lower layer which indicates the presence of Steroids and formation of yellow colored lower layer indicates the presence of triterpenoids.

IV. 2. 3. 7. Quinines

To the test solution added sodium hydroxide solution which gives blue, green and red precipitate within short time if quinine present.

IV. 2. 3. 8. Alkaloids

IV. 2. 3. 8.1. Mayer's test

To the 1 mL of extract, added 1 mL of Mayer's reagent (Potassium mercuric iodide solution).

IV. 2. 3. 8. 2. Wagner's test

To the 1 mL of extract added 2 mL of Wagner's reagent (iodine in potassium iodide) and observed for the formation of reddish brown precipitate which indicates the presence of alkaloids.

IV. 2. 3. 9. Anthraquinones

To 150mg of solvent free extract, 2 mL of water followed by few drops of dilute sulphuric acid is added and allowed to heat on a water bath for 10mins followed by that 1 mL of dilute ammonia was added (Siddiqui and Ali, 1997).

IV. 2. 4. ANTIBACTERIAL ACTIVITY

IV. 2. 4. 1. Test organisms

The test microorganisms used in this study: *Staphylococcus aureus* (NCIM: 2127), *Escherichia coli* (NCIM: 2343), *Salmonella typhi* (NCIM: 2263), *Salmonella typhimurium* (NCIM: 2501), *Klebsiella pneumoniae* (NCIM: 2957), *Bacillus cereus* (NCIM: 2155). The bacterial cultures of these microbes were revived in Nutrient broth medium (Appendix 4.1b) and incubated at 37°C for 48 hours. Each bacterial culture was further maintained at 37°C on nutrient agar slants at 2-8°C and nutrient broth after every 48 hours of transferring.

IV. 2. 4. 2. Agar-well diffusion method for antibacterial activity assay

Nutrient agar medium (Appendix 4.1) was used as bacterial culture medium in the antibacterial assays. The antibacterial activity of the crude extracts was determined in accordance with the agar-well diffusion method described by Irobi *et al.* (1994). The bacterial isolates were first grown in a nutrient broth for 18 h before use. The petri plates were autoclaved at 121°C for 15 minutes and were cooled under laminar air flow. 20 mL of media was added into each sterile Petri dish and solidified. 100µl of the cell suspensions were spread on nutrient agar media plates. Wells were then made in the agar using a sterile 3 mm diameter cork borer. Approximately 30µl of the crude extract was introduced into each wells, kept at room temperature for about 2 h and then incubated at 37°C for 24 h. Control without plant extracts and reference control using standard antibiotic (Ampicillin 20mg/mL) were included in the assay. The plates were observed for zones of inhibition after 24 h and antibacterial activity was determined by measuring the diameters of zone of growth inhibition. The growth inhibition caused by the extract was corrected based on control and compared with those of reference control. The experiments were conducted in triplicate. The data was expressed as mean and standard error (M±SE).

IV. 2. 4. 3. Minimum inhibitory concentration (MIC)

The MIC estimation of the extract was determined using the methods of Gunasekaran (2005). An aliquot (100µl) of the extract solution at concentration of 10 mg/ mL was added to 900µl of presterilized nutrient broth. Subsequently, 100µl from the first test tube was transferred to the second test tube and this continued up to the sixth test tube. Thereafter, 100µl of 24 h of each test bacterium (1.0 x 10^6cells/mL) was inoculated into each test tube and mixed thoroughly. The test tubes were then incubated at 37°C for 24 h.

IV. 2. 5. THIN LAYER CHROMATOGRAPHY (TLC)

In order to analyse variations in chemical constituents, extracts from the callus and regenerated plants which showed high bioactivity and from the mother plant were subjected to thin layer chromatographic analysis. Thin layer chromatography was performed on readymade plates (Merck plates silicagel $60F_{254}$, 20×20 dimension) by applying 10µl of the extract, with toluene: acetone: aceticacid (9:1:0.5) (Vandana *et al.*, 2010), as mobile phase. The separated components were visualized under visible and ultraviolet light (365 and 254nm) using UVi tech of Genei and the Retention values (Rf value) were calculated.

IV. 2. 5. 1. BIOAUTOGRAPHIC OVERLAY ASSAY

Bioautographic agar-overlay assay was done with TLC plate of plant extracts showing significant antimicrobial activity. For bioautographic analysis, developed TLC plates were dried to remove the solvent and placed over nutrient agar plates. It was overlayed with soft agar seeded with a concentrated suspension of actively growing *B. cereus* cells and incubated at 37^0C for 24 hours. The plates were then sprayed with aqueous solution (1mg/mL) of 2, 3, 5-triphenyltetrazolium chloride and incubated for 24 hours. The inhibition zones appeared colorless against a purple background. Spots showing any inhibition were noted and

compared with the Retention factor (Rf) of the related spots on the reference TLC plate. All samples were tested in triplicate and the tests were repeated thrice.

IV. 3. 1. VARIATION IN PHYSICAL PROPERTIES OF EXTRACTS

The physical properties such as colour and viscosity of the extracts were found to vary with different solvents and sources used for extraction.

IV. 3. 1.1. Variation in physical properties of extracts from callus, leaves of *Jatropha curcas* mother plant and *in vitro* regenerated plantlets

Ethanol, methanol and aqueous extracts of callus as well as the leaves of regenerants and mother plant were odorless and slightly viscous. The color of the extracts varied from pale brown to dark green as seen in Table 4.1.

Table 4.1: Variation in the colour of extracts from *J. curcas* callus, regenerated plantlets and mother plant

Solvents	M.P Leaf	I.G.P Leaf	C	M.P Petiole	I.G.P Petiole	M.P Stem	I.G.P Stem	M.P Root	I.G.P Root
Ethanol									
Methanol									
Aqueous									

M.P- Motherplant, I.G.P.-*In vitro* generated plant, L-Leaf, P- Petiole, S-Stem, R- Root, C- Callus

IV. 3. 1.2. Variation in physical properties of extracts from petiole of *J. curcas* mother plant and *in vitro* regenerated plantlets

Ethanol, methanol and aqueous extracts were found to be slightly viscous and odorless. Ethanol extract of the mother plant was green, and methanol extract was dark green. Both the extracts from regenerated plantlets were yellowish green in color. Aqueous extract was reddish brown color in both the sample (Table 4.1).

IV. 3. 1.3. Variation in physical properties of extracts from stem of *J. curcas* mother plant and *in vitro* regenerated plantlets

All extracts of mother plant and regenerated plantlets showed similar physical properties. They were viscous and odorless. The Ethanol extract was yellow, methanol extract was light yellow and the water extract was dark yellow in color.

IV. 3. 1.4. Variation in physical properties of extracts from root of *J. curcas* mother plant and *in vitro* regenerated plantlets

The ethanol, methanol and aqueous extracts of mother plant and regenerated plantlets showed similar characteristics, except color. The water extract was reddish brown, whereas ethanol and methanol extracts were yellow in color in both mother plant and regenerated plantlets (Table 4.1).

IV. 3. 2. PHYTOCHEMICAL SCREENING OF EXTRACTS

IV. 3. 2. 1. Phytochemical screening of extracts from callus, leaves of *J. curcas* mother plant and *in vitro* regenerated plantlets

The presence of flavanoids, saponins, tannins, anthroquinones and alkaloids could be detected in ethanol, methanol and aqueous extract from callus and leaves of *J. curcas*. Sterols, terpenoids and steroids could not be detected in any of the extracts. No variation could be detected in the constituents of the extracts from leaves of mother plant and tissue culture regenerant. However glycosides and phenols could not be detected in extracts from callus regenerated from leaf discs although they were present in leaf extracts.

Table 4.2: Phytochemical screening of extracts from *J. curcas* mother plant, *in vitro* regenerated plantlet and callus.

Secondary metabolite		Flavonoids	Glycosides	Phenols	Saponins	Sterols	Tannins	Terpenoids	Quinones	Anthraquinones	Steroids	Alkaloids
MP Ethanol extract	L	+	+	+	+	-	+	-	+	+	-	+
	P	+	-	+	+	+	+	+	+	+	+	+
	S	+	-	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+
IGP Ethanol extract	L	+	+	+	+	-	+	-	+	+	-	+
	P	+	-	+	+	+	+	+	+	+	+	+
	S	+	-	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+
Ethanol extract	C	+	-	-	+	-	+	-	+	+	-	+
MP Methanol extract	L	+	+	+	+	-	+	-	+	+	-	+
	P	+	-	+	+	+	+	+	+	+	+	+
	S	+	-	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+
IGP Methanol extract	L	+	+	+	+	-	+	-	+	+	-	+
	P	+	-	+	+	+	+	+	+	+	+	+
	S	+	-	+	+	+	+	+	+	+	+	+
	R	+	+	+	+	+	+	+	+	+	+	+
Methanol extract	C	+	-	-	+	-	+	-	+	+	-	+
MP Water extract	L	+	+	+	+	-	+	-	+	+	-	+
	P	+	-	-	+	-	+	-	+	+	-	-
	S	+	-	-	+	-	+	-	+	+	-	-
	R	+	+	+	+	+	+	+	+	+	+	+
IGP Water extract	L	+	+	+	+	-	+	-	+	+	-	+
	P	+	-	-	+	-	+	-	+	+	-	-
	S	+	-	-	+	-	+	-	+	+	-	-
	R	+	+	+	+	+	+	+	+	+	+	+
Water extract	C	+	-	-	+	-	+	-	-	+	-	+

M.P- Motherplant, I.G.P.-*In vitro* generated plant, L-Leaf, P- Petiole, S-Stem, R- Root, C- Callus

IV. 3. 2. 2. Phytochemical screening of extracts from petiole of *J. curcas* mother plant and *in vitro* regenerated plantlets

Flavonoids, phenolic compounds, saponins, sterols, steroids, terpenoids, tannins, quinines, anthraquinones and alkaloids were found to be present in ethanol and methanol extracts of petiole of mother plant and regenerant where as phenol, terpenoids and sterols were not detected in aqueous extract (Table 4.2). Glycosides were absent in all the extracts. There was no variation between phytochemical profile in mother plant and *in vitro* generated plant for the tested secondary metabolites.

IV. 3. 2. 3. Phytochemical screening of extracts from stem of *J. curcas* mother plant and *in vitro* regenerated plantlets

The qualitative estimation of the phytochemicals in the extracts of stem of *J. curcas* revealed the presence of all the tested secondary metabolites except glycosides as shown in Table 4.2. The phytochemical profile found to be similar in mother plant and *in vitro* regenerants. Nevertheless phenols, terpenoids, steroids and alkaloids could not be detected in aqueous extracts of both mother plant and *in vitro* regenerants.

IV. 3. 2. 4. . Phytochemical screening of extracts from root of *J. curcas* mother plant and *in vitro* regenerated plantlets

All the tested phytochemicals were found to be present in root extract of *Jatropha curcas* mother plant and *in vitro* regenerants (Table 4.2)

IV. 3. 3. ANTIBACTERIAL ACTIVITY OF EXTRACTS

IV. 3. 3. 1. Antibacterial activity of extracts from callus, leaves of *Jatropha curcas* mother plant and *in vitro* regenerated plantlets

The ethanol extract was found to be more effective (zone of inhibition 4-11mm) than the methanol extract (zone of inhibition 2–5mm) against all the

organisms tested (Figure 4.1). The water extract showed low antibacterial activity with inhibition zones ranging between 0 and 3 mm for different bacteria tested. When comparing the extracts from mother plant and *in vitro* regenerant, the ethanol extracts of the regenerant was found to show greater inhibition of *S. aureus, S. typhi* and *E. coli* with zones of inhibition, 11, 5.6 and 7.6mm respectively. Methanol extract from the leaf of regenerant showed higher inhibitory zone than that of the mother plant to *E. coli* whereas methanol extract from callus inhibited growth of *S. aureus* than that of mother plant and regenerant. Aqueous extract of the callus seemed to have higher resistance than aqueous extract from leaf of mother plant and regenerant.

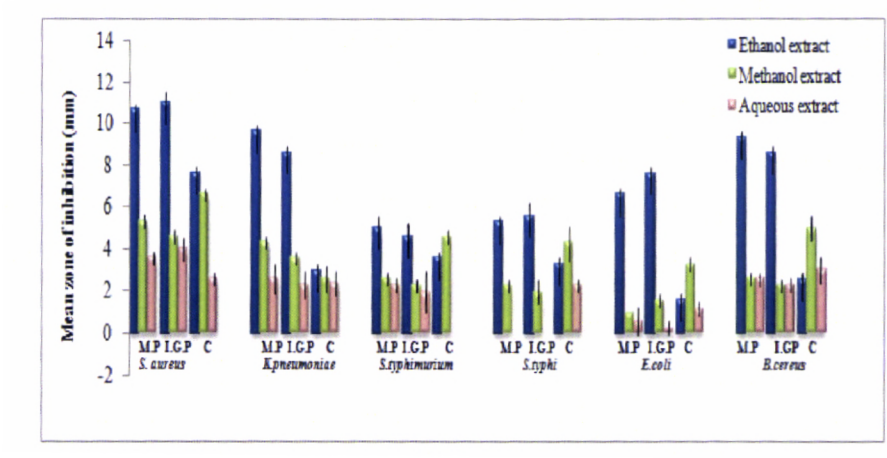


Figure 4.1: Antibacterial activity of extracts from callus, leaves of *J. curcas* mother plant and *in vitro* regenerated plantlets. Values represent mean ± SE.

MP- Mother Plant, IGP- *In vitro* Generated Plant, C- Callus

IV. 3.3.2. Antimicrobial activity of extracts from petiole of *J. curcas* mother plant and *in vitro* regenerated plantlets

In the agar well diffusion assay, petiole extract of *J. curcas* mother plant and regenerant showed activity against all the tested bacteria. It is evident from Figure 4.2 that the ethanol extract showed the maximum activity compared to the other two extracts. The inhibitory action of ethanol extract from petiole of regenerant against *K. pneumoniae, S. typhimurium, S. typhi, E. coli and B. cereus* was higher than that of the mother plant extract. Methanol extract of the regenerant showed activity against *S. aureus, S. typhimurium* and *B. cereus* than the same of mother plant petiole.

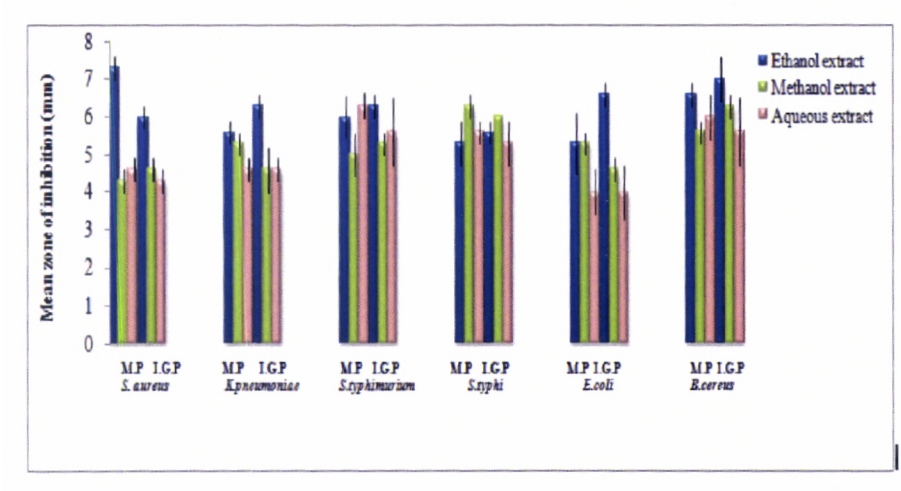


Figure 4.2: Antimicrobial activity of extracts from petiole of *J. curcas* mother plant and *in vitro* regenerated plantlets. Values represent mean ± SE.

MP- Mother Plant, IGP- *In vitro* Generated Plant

IV. 3. 3. 3. Antimicrobial activity of extracts from stem of *J. curcas* mother plant and *in vitro* regenerated plantlets

Ethanol, methanol and aqueous stem extracts of *J. curcas* mother plant and regenerant used for the test showed good resistance against the growth of 6

bacteria tested. The zone of inhibition was 7.6 mm by regenerant ethanol extract towards *K. pneumoniae*, while it was 7.3 by mother plant extract (Figure 4.3). The inhibitory action of methanol extract from the stem of regenerant against *E. coli* and *B. cereus* was higher than the mother plant extract. Methanol extracts of both the samples showed same zone diameter (4.6mm) against *S. typhi*. Activity of Aqueous extract of *S. typhimurium* and *B. cereus* was higher than that of mother plant.

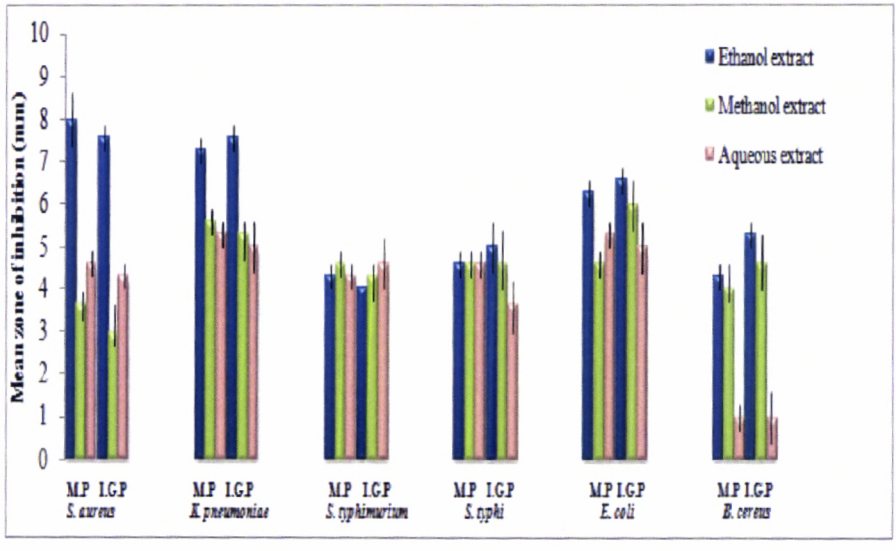


Figure 4.3: Antibacterial activity of extracts from stem of *Jatropha curcas* mother plant and *in vitro* regenerated plantlets. Values represent mean ± SE.

MP- Mother Plant, IGP- *In vitro* Generated Plant

IV. 3. 3. 4. Antimicrobial activity of extracts from root of *Jatropha curcas* mother plant and *in vitro* regenerated plantlets

Root extract found to be highly active against all the 6 bacteria tested, in compared to other stem, petiole and leaf extracts. Root ethanol extract of regenerant showed higher resistance to *S. aureus* (20.3 mm) and *B. cereus* (24.7 mm) than that of mother plant (18.0 mm and 23.3 mm respectively). Methanol

stem extract of *J. curcas* regenerant also showed higher activity against *S. typhimurium, S. typhi* and *E. coli* than mother plant extract. The inhibitory action of aqueous extract from root of regenerant against *S. aureus* was same as that of mother plant and they responded slightly higher against all organisms except *K. pneumoniae* (Figure 4.4).

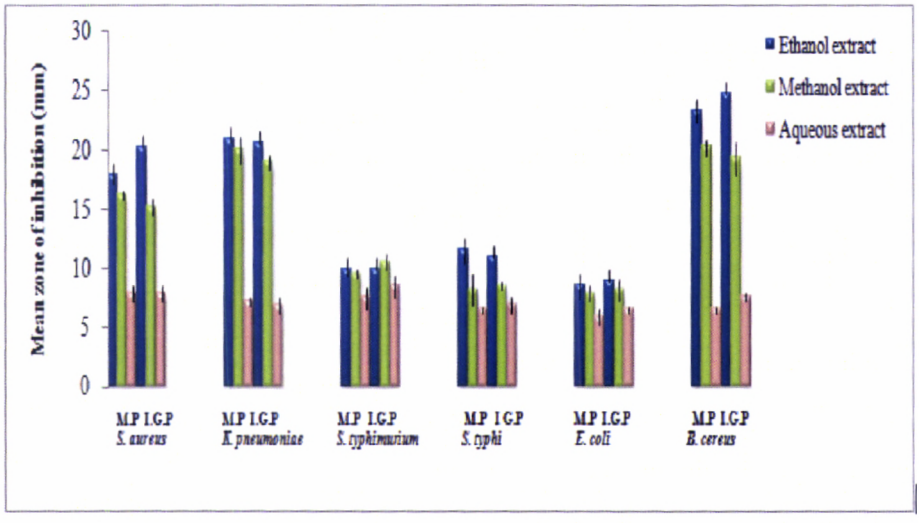


Figure 4.4: Antibacterial activity of extracts from root of *Jatropha curcas* mother plant and *in vitro* regenerated plantlets. Values represent mean ± SE.

MP- Mother Plant, IGP- *In vitro* Generated Plant

IV. 3. 4. MINIMUM INHIBITORY CONCENTRATION OF EXTRACTS

Ethanolic extract from leaf, petiole, stem and root of *J. curcas* exhibited considerable antimicrobial activity, than methanol and aqueous extracts, hence minimum inhibitory concentration (MIC) of extracts from mother plant and *in vitro* regenerated plantlets analyzed with 6 bacterial strains. MIC of Ampicillin control tested against the same organisms depicted in the table 4.3 for comaparison with the test extracts

IV. 3. 4. 1. Minimum inhibitory concentration of extracts from callus generated from leaf explants and leaves of *J. curcas* mother plant and *in vitro* regenerated plantlets

The minimum inhibitory concentration (MIC) of the leaf ethanol extract from mother plant and *in vitro* generated plants for different organisms was found to be similar in both. MIC for *S. aureus, K. pneumonia and B. cereus* was 1.0mg/mL, whereas for others it was 10.0 mg/L. MIC of callus extract was found to be higher against *S. aureus, K. pneumoniae, E. coli* and *B. cereus* than extracts from leaves of *Jatropha curcas* mother plant and *in vitro* regenerants (Table 4.3).

Table 4.3: MIC of ethanol extracts from callus generated from leaf explants leaves, petiole, stem and root of *J. curcas* mother plant and *in vitro* regenerated plantlets

Test bacteria	M.P L (mg mL)	I.G.P L (mg mL)	C (mg mL)	M.P P (mg mL)	I.G.P P (mg mL)	M.P S (mg mL)	I.G.P S (mg mL)	M.P R (mg mL)	I.G.P R (mg mL)	Ampicillin (mg mL)
Staphylococcus aureus	1.0	1.0	10.0	1.0	1.0	1.0	1.0	0.1	0.1	2×10^{-3}
Klebsiella pneumoniae	1.0	1.0	10.0	10.0	10.0	10.0	10.0	1	1	2×10^{-3}
Salmonella typhimurium	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10	10	2×10^{-3}
Salmonella typhi	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10	10	2×10^{-3}
Escherichia coli	10.0	10.0	100.0	10.0	10.0	10.0	10.0	10	10	2×10^{-3}
Bacillus cereus	1.0	1.0	10.0	1.0	1.0	1.0	1.0	0.1	0.1	2×10^{-3}

M.P- Motherplant, I.G.P.-*In vitro* generated plant, L -Leaf, P- Petiole, S-Stem, R- Root, C- Callus

IV. 3. 4. 2. Minimum inhibitory concentration of petiole extracts of *J. curcas* mother plant and *in vitro* regenerated plantlets

In the MIC analysis, *S aureus* and *B cereus* required the extract at MIC, 1.0 mg/L, where as bacterial growth was observed with 1.0 mg/mL ethanolic extract and found that 10 mg/mL of the extract is necessary for completely inhibiting the bacterial growth. (Table. 4.3).

IV. 3. 4. 3. Minimum inhibitory concentration of stem extracts of *J. curcas* mother plant and *in vitro* regenerated plantlets

The minimum inhibitory concentration (MIC) of the *J curcas* stem ethanol extract of mother plant and *in vitro* regenerants for organisms, *Staphylococcus aureus and Bacillus cereus* were found to be 1.0 mg/mL (Table 4.3). Ampicillin showed equivalent activity against *Staphylococcus aureus* and *Bacillus cereus* at 0.2 mg/L and 0.002 mg/L concentration The activity of the extracts inhibited the growth of other organisms at MIC values lower or equal to 10.0 mg/mL.

IV. 3. 4. 4. Minimum inhibitory concentration of root extracts of *J. curcas* mother plant and *in vitro* regenerated plantlets

The minimum inhibitory concentration (MIC) of the *J curcas* root ethanol extract of mother plant and *in vitro* regenerants for organisms, *Staphylococcus aureus and Bacillus cereus* were found to be 0.1 mg/mL (Table 4.3). MIC for resisting *Klebsiella pneumoniae* was 1.0 mg/L for root extracts of *Jatropha curcas* mother plant and *in vitro* regenerants. Ampicillin showed equivalent activity against *Staphylococcus aureus* and *Bacillus cereus* at 0.2 mg/L and 0.002 mg/L concentration. The activity of extracts inhibited the growth of other organisms at MIC values lower or equal to 10.0 mg/mL.

IV.3.5. THIN LAYER CHROMATOGRAPHY ANALYSIS OF EXTRACTS

IV. 3. 5. 1. Thin layer chromatography analysis of of extracts from callus, leaves of *J. curcas* mother plant and *in vitro* regenerated plantlets

Thin layer chromatography analysis of *Jatropha curcas* leaf ethanolic extracts of mother plant and *in vitro* generated plants showed that they have 4 constituents, where as callus showed 5 fractions. The components showed Rf values, 0.28, 0.33, 0.48 and 0.57 in mother plant, and 0.28, 0.31, 0.46 and 0.63 in regenerant leaf ethanol extract (Table 4.4 and Figure 4.5).

Table 4.4: Thin layer chromatography analysis of ethanol extracts from callus, leaves of *J. curcas* mother plant and *in vitro* regenerated plantlets with solvents Toluene: acetone: acetic acid (9:1:0.5)

Ethanol Extract	No: of components	Color of the band	Rf value	inhibitory effect on *S. aureus*.
Mother plant leaf	4	Orange	0.28	-
		Orange	0.33	-
		Orange	0.48	-
		Orange	0.57	++
Callus	5	Blue	0.24	+
		Orange	0.31	+
		Orange	0.37	-
		Orange	0.53	++
		Orange	0.57	
Regenerated plant leaf	4	Orange	0.28	+
		Orange	0.31	+
		Orange	0.46	-
		Orange	0.63	+

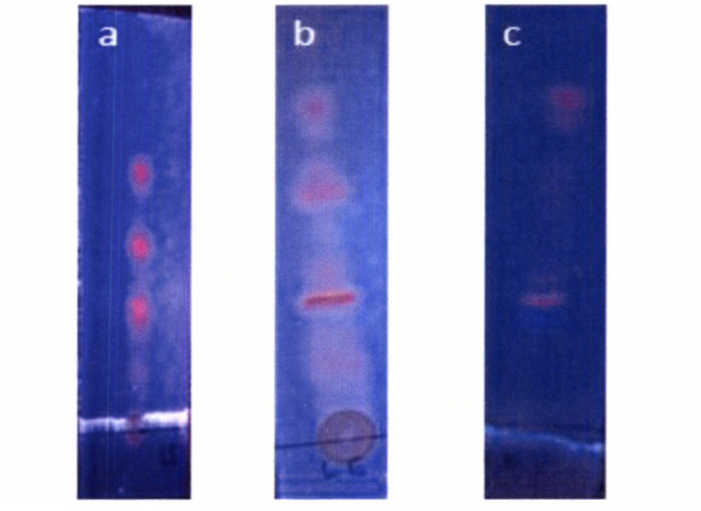


Figure 4.5: Thin layer chromatography analysis of ethanol extracts from leaves of *J. curcas* (a) mother plant (b) *in vitro* regenerated plantlets and (c) callus generated from leaf explants

IV. 3. 5. 2. Thin layer chromatography analysis of ethanol extracts from petiole of *J. curcas* mother plant and *in vitro* regenerated plantlets

Eight and nine different components were found to be present in the ethanolic extract of *Jatropha curcas* petiole in motherplant and regenerant respectively, as depicted in table 4.The chromatogram was visible under uv at 365nm (Table 4.5and Figure 4.6).

Table 4.5: Retention factor of thin layer chromatography analysis of ethanol extracts from petiole of *J. curcas* mother plant and *in vitro* regenerants with solvents Toluene: acetone: acetic acid (9:1:0.5)

Ethanol extract	No: of components	Color of the band	Rf value	Inhibitory effect on *B. cereus*
Mother plant	8	Blue	0.19	+
		Red	0.20	+
		Red	0.48	+
		Blue	0.52	-
		Red	0.55	-
		Blue	0.65	-
		Red	0.74	-
		Red	0.87	-
Regenerated plant	9	Blue	0.19	+
		Red	0.23	+
		Blue	0.30	+
		Red	0.47	
		Blue	0.53	-
		Red	0.57	-
		Blue	0.60	-
		Red	0.73	-
		Red	0.85	-

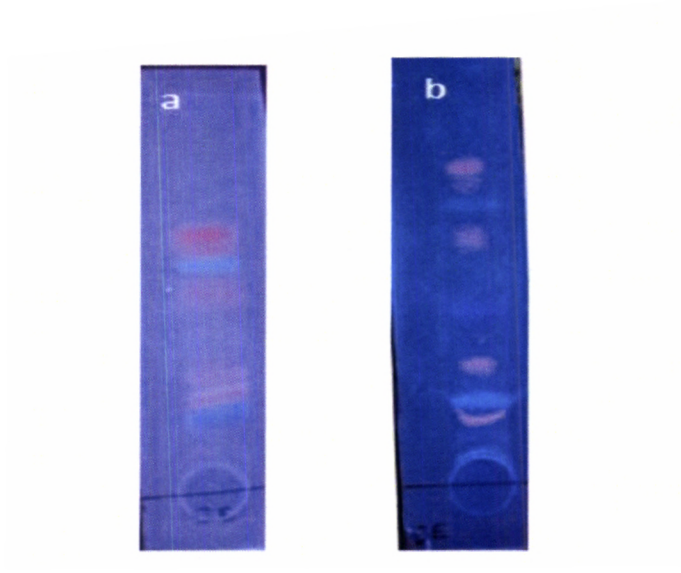


Figure 4.6: Thin layer chromatography analysis of ethanol extracts from petiole of *J. curcas* (a) mother plant and (b) *in vitro* regenerants

IV. 3. 5. 3. Thin layer chromatography analysis of ethanol extracts from the stem of *J. curcas* mother plant and *in vitro* regenerants

Seven and Nine different components were observed in the ethanolic extract of *J. curcas* stem of mother plant and regenerant respectively. The Rf values are summarized in table 4.6. When visualized under UV at 365nm the fractions were found to be with different color pattern (Figure 4.7).

Table 4.6: Retention factor of thin layer chromatography analysis of ethanol extracts from the stem of *J. curcas* mother plant and *in vitro* regenerants with solvents Toluene: acetone: acetic acid (9:1:0.5)

Ethanol extract	No: of components	Color of the band	Rf value	Inhibitory effect on *S. aureus*
Mother plant	7	Blue	0.15	+
		Peach	0.27	-
		Peach	0.50	-
		Blue	0.54	-
		Orange	0.61	-
		blue	0.72	-
		Red	0.92	-
Regenerated plant	9	Blue	0.10	+
		Blue	0.12	+
		Orange	0.14	+
		Blue	0.15	+
		Red	0.30	-
		Blue	0.55	-
		Orange	0.64	+
		Blue	0.73	+
		red	0.95	+

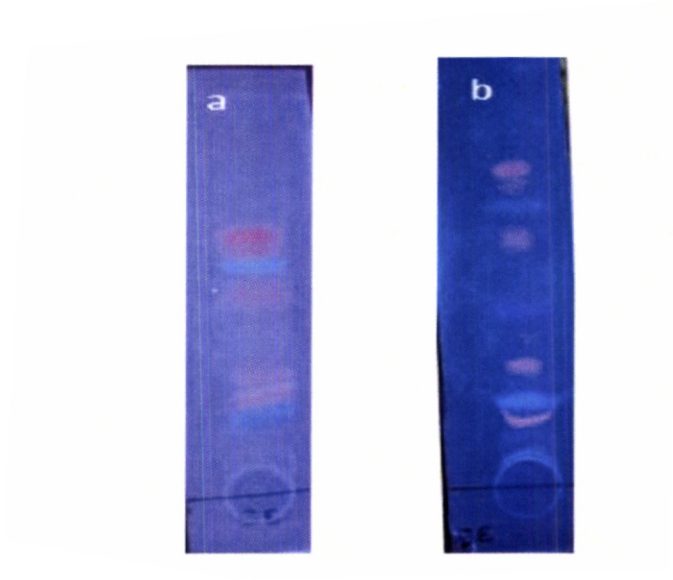


Figure 4.7: Thin layer chromatography analysis of ethanol extracts from stem of *J. curcas* (a) mother plant and (b) *in vitro* regenerants

IV. 3. 5. 4. Thin layer chromatography analysis of ethanol extracts from the root of *J. curcas* mother plant and *in vitro* regenerants

Thin layer chromatography analysis of ethanol extracts from root of *J. curcas* mother plant and *in vitro* regenerants showed 9 fractions and there was no variation between them in the TLC profile (Table 4.7 and Figure 4.8).

Table 4.7: Retention factor of ethanol extracts from the root of *J. curcas* mother plant and *in vitro* regenerants with solvents Toluene: acetone: acetic acid (9:1:0.5)

Ethanol extract	No: of components	Colour of the bands	Rf value	Inhibitory effect on *S. aureus*
Mother plant	9	Blue	0.10	+
		Blue	0.17	+
		Blue	0.20	-
		Blue	0.25	+
		Blue	0.30	+
		Blue	0.35	-
		Blue	0.42	+
		Blue	0.62	+
		Blue	0.79	+
Regenerated plant	9	Blue	0.11	+
		Blue	0.16	+
		Blue	0.22	-
		Blue	0.25	+
		Blue	0.31	+
		Blue	0.37	-
		Blue	0.42	+
		Blue	0.65	+
		Blue	0.80	+

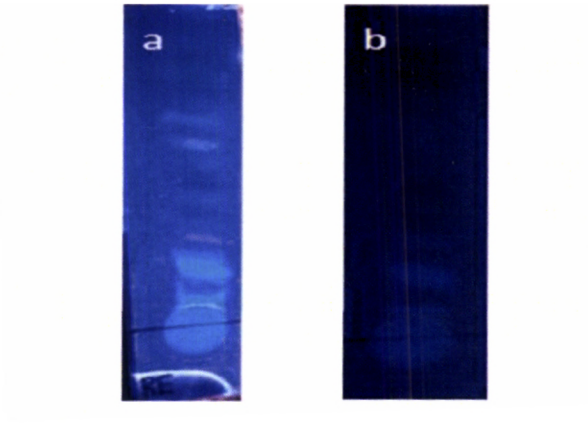


Figure 4.8: Thin layer chromatography analysis of ethanol extracts from root of *J. curcas* (a) mother plant and (b) *in vitro* regenerants

IV.3.6. BIOAUTOGRAPHIC OVERLAY ANALYSIS OF EXTRACTS

IV.3.6.1. Bioautographic overlay analysis of ethanol extracts from the callus generated from leaf explants and leaves of *J. curcas* mother plant and *in vitro* regenerated plantlets

Bioautographic overlay analysis of *J. curcas* leaf extracts of mother plant and *in vitro* generated plants, showed maximum antimicrobial activity against *S. aureus*. The fractions with Rf value 0.57 in mother plant and 0.63 *in vitro* regenerated plant has inhibitory effect on *S. aureus*. Apart from that the fractions with Rf values 0.28 and 0.31 in regenerants were also found to be given a slight clear zone on assay plate (Table 4.4). Similarly the callus extract also showed clear zones at Rf values 0.57, 0.24 and 0.31

IV.3.6.2. Bioautographic overlay analysis of petiole ethanol extracts of *J. curcas* mother plant and *in vitro* regenerated plantlets

Table 4.5 shows the TLC bioautographic results of *J. curcas* petiole ethanol extract. The bioautography revealed zones of growth inhibition for *B. cereus*, where as in other bacteria the zones of inhibition were less visible. Clear zone observed in the Rf zone lies between 0.19-0.48 in both the sample.

IV.3.6.3. Bioautographic overlay analysis of ethanol extracts from the stem of *J. curcas* mother plant and *in vitro* regenerated plantlets

The bioautography revealed clear zones of bacterial growth inhibition with purple background for *S. aureus*, where as in other bacteria the zones of inhibition were less visible. When the assay results of mother plant and *in vitro* regenerants were compared, the later showed two clear zones at Rf values ranging from 0.10 to 0.15 and 0.64 to 0.95, while mother plant extract developed only one inhibitory zone (Table 4.6)

IV.3.6.4. Bioautographic overlay analysis of root extracts of *J. curcas* mother plant and *in vitro* regenerated plantlets

The result showed clear zones of bacterial growth inhibition for *S.aureus*, at Rf ranges 10-25, 30-37, 79-80 when extracts from root of mother plant and *in vitro* regenerants tested (Table 4.7).

IV. 4. 1. Ethanol extract from all plant parts tested had higher bioactivity compared to methanol and aqueous extracts

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process and potential health hazard of the extractants (Eloff, 1998). Ethanol has the ability to extract more components, this may be the reason for the presence of large number of phytochemicals in ethanol extract (Arekemase *et al.*, 2011).

Plant extracts using organic solvents have been found to give more consistent antimicrobial activity compared to water extract (Tiwari *et al.*, 2011). Study by Adebayo and Kofi, (2011) revealed that the ethanol extract of the plant has greater antibacterial potential than the aqueous and petroleum ether extracts. The higher activity of the ethanolic extracts as compared to the aqueous extract can be attributed to the presence of higher amounts of polyphenols as compared to aqueous extracts. Ethanolic extracts are more efficient in cell wall and seed degradation and cause polyphenols to be released from cells. The decrease in activity of aqueous extract can be ascribed to the enzyme polyphenol oxidase, which degrades polyphenols in water extracts, whereas in methanol and ethanol they are inactive (Lapornik *et al.*, 2005). The higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70% due to its higher polarity than pure ethanol (Bimakr *et al.*, 2011). Additionally, ethanol possibly penetrates the cellular membrane easily to extract the intracellular ingredients

from the plant material (Wang, 2010). Methanol is more polar than ethanol but due to its cytotoxic nature, it is unsuitable for extraction in certain kind of studies (Tiwari *et al.*, 2011).

IV. 4. 2. Presence of pharmaceutically important phytochemicals detected in the plant extracts

Flavonoids, glycosides, phenols, saponins, sterols, tannins, terpenoids, quinines, anthroquinones, steroids and alkaloids were found to be present in different plant parts and there was no variation in regenerated plants compared to mother plant, whereas variation was observed in the extract from callus. Phytochemical screening showed that *J. curcas* extract contains tannins, flavonoids, alkaloids, anthraquinones and phenols (Ekundayo *et al.*, 2011). Of these tannins have been found to form irreversible complexes with proline rich protein (Shimada, 2006) resulting in the inhibition of cell protein synthesis. Tannins are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery (Dharmananda, 2003). These observations therefore support the use of *J. curcas* in herbal cure remedies. Li and Wang, (2003) reviewed the biological activities of tannins and observed that tannins have anticancer activity and can be used in cancer prevention, thus suggesting that *J. curcas* has potential as a source of important bioactive molecules for the treatment and prevention of cancer. Parekh and Chanda, (2007) reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflamed or ulcerated tissues. Alkaloids present in *J. curcas* have led to the development of powerful pain killer (Kam and Liew, 2002). One of the most common biological properties of alkaloids is their toxicity against cells of foreign organisms. These activities have been widely studied for their potential use in the elimination and reduction of human cancer cell lines (Nobori *et al.*, 1994).

Another important phytochemical detected was glycosides, which have long been used as cardio tonic, also in nephrological diseases. They have been shown to be useful in managing infections. Phenolic compounds found in *J. curcas* are found to reduce the risk of heart disease, certain types of cancer and decrease cholesterol level. Phenolic toxicity to microorganisms is due to the site(s) and number of hydroxyl groups present in the phenolic compound (Urs and Dunleavy *et al.*, 1975; Scalbert, 1991). Flavonoids are most commonly known for their antioxidant activity. The presence of flavonoids and tannins in the all the extract is likely to be responsible for the free radical scavenging activity (Potterat, 1997). This accounts for their ability as UV –protectants. Flavonoids have been referred to as nature's biological response modifiers because of strong experimental evidence of their ability to modify the body's reaction to allergies, virus and carcinogens (Denwick, 2002). They show anti-allergic, anti-inflammatory, antimicrobical and anticancer activity (Balch and Balch, 2000; Ekam and Ebong, 2007). Analgesic, anti-angiogenic, cytostatic and antioxidant properties of the plant extract have been reported by Hodek *et al.* (2002).

Just *et al.*, (1998) revealed the inhibitory effect of saponins on inflamed cells. Saponin was found to be present in *J. curcas* extracts and has supported the usefulness of this plant in managing inflammation. Saponins possess hypocholesterolemic and antidiabetic properties (Rupasinghe *et al.*, 2003). Steroidal compounds present in *J. curcas* extracts are of importance and interest due to their relationship with various anabolic hormones including sex hormones (Okwu, 2001). Quinlan *et al.*, (2000) worked on steroidal extracts from some medicinal plants which exhibited antibacterial activities on some bacterial isolates. Neumann *et al.* (2004) confirmed the antiviral property of steroids. Steroids and triterpenoids showed analgesic properties (Sayyah, 2004; Malairajan *et al.*, 2006). The steroids and saponins are also responsible for central nervous system activities (Argal and Pathak, 2006).

The presence of these bioactive components is a confirmation of the significance of *J. curcas*, serving as an ingredient in the traditional medicinal system.

IV. 4. 3. Root extract had higher bioactivity compared to leaf, petiole and stem extracts

In a study by Sharma *et al.*, (2012) it was found that the root of *J. curcas* has high activity. The alcoholic extract of roots exhibited analgesic activity and significant reduction in pyrexia that was comparable to standard drugs pantazocine and paracetamol respectively (Nayak and Patel, 2010). The roots are used in decoction as a mouthwash for bleeding gums, toothache, eczema, ringworm, scabies and to cure dysentery and venereal diseases like gonorrhoea. It is also reported that the root methanol extract exhibit anti-diarrhoeal activity in mice through inhibition of prostaglandin biosynthesis and reduction of osmotic pressure (Oliver-Bever, 1986). The alcoholic extract of root exhibited higher systemic and significant anti-inflammatory activity in acute carrageenan-induced rat paw edema than stem and leaf (Nayak and Patel, 2010). *J. curcas* root extract consists of β-sitosterol and its β-D-glucoside, marmesin, propacin, the curculathyranes A and B and the curcusones A–D, diterpenoids jatrophol and jatropholone A and B, the coumarin tomentin, the coumarino-lignan jatrophin as well as taraxerol (Naengchomnong *et al.*, 1986; 1994).

IV. 4. 4. Antibacterial activity against *S. aureus, K. pneumoniae, S. typhimurium, S. typhi, E. coli and B. cereus* was higher in extracts of callus and regenerated plants than from mother plants

An enhancement in the antimicrobial activity of extracts from callus and regenerated plants was observed. The inhibitory activity of plant extract is largely dependent on the concentration, parts of the plant used and the microbes tested (Kalimuthu *et al.*, 2010). Stem ethanol extract had the highest antibacterial activity

than others. The lack of activity of the water extract against most bacterial strains investigated in this study is in agreement with previous works which showed that aqueous extracts of plant generally showed little or no antibacterial activity. (Aliero *et al.*, 2006 ; Koduru *et al.*, 2006 ; Ashafa *et al.*, 2008 ; Aiyegoro *et al.*, 2008; Oseni *et al.*, 2011). Adebayo and Kofi, (2011) reported that root, stem bark and leaf ethanol extracts demonstrated comparable antibacterial activities against *S. typhi* and *E. coli.*

The compounds found to be present in phytochemical screening in this study are known to be biologically active and therefore may aid in the antimicrobial activities of *J. curcas*. This enhancement in the activity might be due to the accumulation of active metabolites in the cell lines of callus cultures (Nezbedová *et al.*, 1999). Similar enhanced activity of the callus extracts have been reported in *Heterostemma tanjorense*, *Eclipta alba* and *Bixa orellana* (Kathiresan and Ravikumar, 1997; Lakshmi *et al.*, 1999; Sagar and Zafar *et al.*, 2000; Castello *et al.*, 2002). However, the extent of enhancement depends on many factors like type and composition of media, plant growth regulators and culture conditions; among which plant growth regulators play vital role in the production of the active compounds (Morris *et al.*, 1985). However, there is no unanimous opinion about the relation between growth regulators in the media and production of active principle(s) (Nezbedová *et al.*, 1999; Kathiresan and Ravikumar, 1997; Morris *et al.*, 1985). A positive relation between production of active metabolites in the callus and growth regulator (2, 4-D) was reported by Majumder and Jha, (2009).

Antibacterial test results presented showed enhanced antibacterial activity against gram-positive bacteria especially *Bacillus cereus* and *Staphylococcus aureus* in regenerant. The larger zones of inhibition exhibited by the ethanolic extract of *J. curcas* against the test organism may be due to the presence of variety of active compounds induced to produce secondary metabolites such as tannins,

alkaloids, flavonoids and saponins (Abo *et al.*, 2000). In small quantities, anthraquinones act as potent antimicrobial (Lorenzetti, 1964) and antiviral (Sims, 1971) agents.

IV. 4. 5. Variation was detected in number of components present in the extracts from callus and regenerated plants

Number of components in the callus, petiole and stem extracts of regenerated plant was found to be varied from the extracts of motherplant. Regenerants and motherplant of *Withania somnifera* varied in HPTLC band pattern (Shetty and Nareshchandra, 2012). TLC profiling showed the variation in different components in the ethanolic extract and overlay bioautography proved their antibacterial activity against bacterial strain. *In vitro* regenerated plantlets have been reported to produce higher yields of active compounds (Fowke *et al.*, 1994; Mahagamasekera and Doran, 1998; Han *et al.*, 1999). A quantitative HPLC analysis of methanolic extract of leaf and root indicated the enhancement of baicalein -7-o glucoside in *in vitro* raised root and leaf of *Oroxylum indicum* (Gokhale and Bansal, 2010).

CHAPTER V

SUMMARY AND CONCLUSION

Nodal explants containing lateral buds cultured on MS basal medium supplemented with 1.0 mg/L BAP and 0.4 mg/L GA_3 showed best response and developed multiple shoots at frequency of 60.0 % with 3.6 shoots of height 10.0 mm, with number of leaves 6.0. The highest percentage (60.0%) of callus induction from petiole explants was given by 0.6 mg/L IBA and 2.0 mg/L BAP. Callus response was more from leaf discs when compared to petiole. Callus formed from the petiole was soft, friable, white to light green in colour. All the explants tested produced callus in media containing 0.4 mg/L IBA and 1.5 mg/L BAP. This study revealed that adenine sulphate has no significant effect on the *in vitro* development of the plant. As the concentration of IBA increased the compactness of callus increased, and shoot buds were induced.

Histogenetic differentiation in the form of tracheids was observed in calli formed from leaf explants on MS medium containing IBA and BAP. Calli were heterogeneous in nature with wide variations in size and shapes of cells such as spheroidal, ovoid and elongated.

In this study the combination 0.4 mg/L IBA and 2.0 mg/L BAP in MS media was found to be best for indirect regeneration of multiple shoots of significantly higher shoot height (14.20 mm) from leaf explants. When MS medium was supplemented with TDZ, small globular structures, resembling somatic embryos appeared from the lower surface of the leaf discs. The number of shoot buds was found to be higher in 0.4 mg/L IBA and TDZ 2.5 mg/L.

Hormone free medium and water agar was found to be more efficient than hormone supplemented medium for root induction. Water agar medium showed significantly high root induction when compared to full strength MS and ½

strength MS with 0.2 mg/L IBA. The survival rate of the plantlets was 75.0±12.5% during hardening.

By studying the mitotic index in the callus, the efficiency of a medium with particular hormonal concentration on callus induction could be determined. The study revealed that MS media with hormonal addition of 2 mg/L BAP showed the lowest mitotic index, while the highest mitotic index was seen in 0.4 mg/L IBA and 2 mg/L BAP. Therefore hormonal combination 0.4 mg/L IBA and 2 mg/L BAP was found to be best for callus proliferation in *J. curcas* leaf explants.

DNA sequence variation induced by different combinational treatments of auxin (IBA) and cytokinin (BAP) in the callus and regenerated plants could be found in this study. Hormonal combinations 0.2 mg/L IBA and 2.0 mg/L BAP, and 2.0 mg/L BAP alone, could induce highest variation 0.52 ± 0.10 and 0.56 ± 0.19 in callus and regenerated plants respectively. Hormonal combination 0.6 mg/L IBA and 2.0 mg/L BAP induces lower genetic variation in callus and regenerants. DNA sequence changes that were detected in callus did not reflect the variation in regenerated plants. DNA sequence changes detected in the regenerated plants (0.33±0.24) was found to be lower than in the callus (0.38±0.13). This is possibly an indication of heterogeneous nature of the callus with normal and aberrant cells formed in tissue culture but of regenerated plants arising from mostly normal cells in the callus. Among the molecular marker profiles, variation detected by DAMD markers were significantly high ($p<0.05$), when compared to RAPD and ISSR markers. This may be due to high polymorphism in minisatellite region.

Mitotic index and percentage of shoot response decreased with increase in variation index in callus (correlation coefficient -0.540 and -0.642 respectively). Correlation studies showed that the genetic variation induced by various combinational treatments affected the growth and percentage of response in *in vitro* system.

Detection of presence of flavonoids, phenolic compounds, saponins, sterols, steroids, terpenoids, tannins, quinines, anthraquinones and alkaloids in extracts from different parts of *Jatropha curcas* showed its potential as a source of useful drugs. Higher bioactivity was seen in ethanol extracts than the methanol or aqueous extracts. Variation could be detected in the constituents of the extracts from mother plant and regenerants by TLC profiling. Higher antibacterial activity was seen in the extracts from leaf, petiole, stem and root of *J. curcas* regenerants than that of mother plant. The ethanolic extract showed higher antibacterial activity, followed by methanolic and aqueous extract. MIC assay revealed that *S. aureus* and *E. coli* are more susceptible to *J. curcas* extract.

TLC profiling showed the variation in different components in the ethanolic extract. Further studies are required for the large scale production of *J. curcas* secondary metabolites *in vitro,* using suitable elicitors. In the study, the callus induction from the explants is found to be high therefore production of bioactive compound through callus culture can be done. Root extract showed higher antibacterial activity than extracts from other plant parts tested.

The availability of efficient regeneration protocol is highly desirable for crop plants. The plant regeneration protocol reported in the present investigation could be useful for mass production of plants. Since callus culture system offer many advantages as a model system for several biological investigations the present findings in callus could be used in mass propagation and secondary metabolite production.

The study shows that combined molecular analysis can be applied to assess the genetic fidelity of plants produced *in vitro*. This method might be useful for monitoring the stability of *in vitro* germplasm collections and cryopreserved material. Sequence variations observed in regenerants do not necessarily correspond to phenotypic variations in the field; however variation at the DNA sequence level increases the possibility of these changes being observed at the level of the phenotype.

APPENDIX 1

a) Murashige and Skoog Medium (1962)

Components	mg/L in MS
NH_4NO_3	1650
KNO_3	1900
H_3BO_3	6.20
KH_2PO_4	170
$CaCl_2.H_2O$	440
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.2H_2O$	0.025
$MgSO_4.7H_2O$	370
$MnSO_4.2H_2O$	22.3
$ZnSO_4.4H_2O$	8.6
$CuSO_4.5H_2O$	0.025
$FeSO_4.7H_2O$	27.8
Na EDTA	37.3
Myo-Inositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Glycine	2.0
Sucrose	3.0 %
Agar	0.8%
pH	5.6-5.8

1.b. Media for culturing bacteria

- Nutrient agar – 28 g nutrient agar (HiMedia, Mumbai) in 1000 ml distilled water.
- Nutrient broth – 13 g nutrient broth (HiMedia, Mumbai) in 1000 ml distilled water.

Appendix 2.a: ANOVA Table showing significance between the combinations on callus induction from petiole explants by *Post hoc* – tukey test

	Kn-1	Kn-2	Kn+IAA+BA	IBA+BAP+AS	AS+IBA 20.0+0.2	IBA+BAP 1.0+2.0	IBA+BAP 1.0+3.0	IBA+BAP 1.2+2.0	IBA+BAP 1.3+2.0	IBA+BAP 1.3+5.0	IBA+BAP 1.4+1.0	IBA+BAP 1.4+1.5	IBA+BAP 1.4+2.0	IBA+BAP 1.4+2.5	IBA+BAP 1.4+3.0	IBA+BAP 1.4+4.0	IBA+BAP 1.5+5.0	IBA+BAP 1.6+2.0
Kn-1		42.8**	28.3**	24.5**	18.2**	28.9**	13.9*	45.5**	18.2**	16.5**	26.3**	15.3**	32.5**	24.4**	18.5**	25.8**	30**	50**
Kn-2	-42.8**		-14.5**	-18.3**	-24.5**	-14.3**	-28.2**	2.7	-24.5**	-26.3**	-22.5**	-27.5**	-11.3*	-18.4**	-24.3**	-16**	-12.8*	7.2
Kn+IAA+BA 0.5+0.	-28.3**	14.5**		-3.9	-13.1*	3.2	-14.8**	17.1**	-10.1*	-11.9*	3.2	-1.2	4.1	-3.9	-9.1	-1.6	1.7	27.7**
IBA+BAP+AS 0.2+	-24.5**	18.3**	3.9		-6.3	4.1	-10.3*	21**	-6.2	-8.3	-4.1	-9.1	1.1	0.0	-5.9	2.3	5.5	25.5**
AS+IBA 20.0+0.2	-18.2**	24.5**	13.1*	6.3		10.7*	-4.5	27.5**	3.3	-1.7	2.1	-2.9	14.5**	5.2	3.3	6.6	11.8*	31.8**
IBA+BAP 1.0+2.0	-28.9**	14.3**	-3.2	-4.1	-10.7*		-15**	16.9**	-10.7*	-12.1*	-8.2	-13.2*	3.9	-4.1	-10*	-1.8	1.5	21.5**
IBA+BAP 1.0+3.0	-13.9*	28.2**	14.8**	10.3*	4.5	15**		31.9**	4.7	2.3	6.8	1.3	18.9**	10.3*	5.3	13.2*	15.4**	36.4**
IBA+BAP 1.2+2.0	-45.5**	-2.7	-17.1**	-21**	-27.2**	-16.9**	-31.9**		-27.2**	-29**	-25.1**	-30.1**	-13*	-21**	-25.3**	-18.7**	-15.5**	4.5
IBA+BAP 1.3+2.0	-18.2**	24.5**	10.1*	6.2	3.3	10.7*	-4.7	27.2**		-1.8	2.1	-2.9	14.2**	5.2	3.3	6.5	11.8*	31.8**
IBA+BAP 1.3+5.0	-16.5**	26.3**	11.9*	6.0	1.7	12.1*	-2.9	29**	1.8		3.9	-1.1	16**	8.0	2.1	10.7*	13.5*	33.5**
IBA+BAP 1.4+1.0	-26.3**	22.5**	3.2	4.1	-2.1	8.2	-4.8	25.1**	-2.1	-3.9		-5.0	12**	4.1	-1.8	6.4	9.7	29.7**
IBA+BAP 1.4+1.5	-15.3**	27.5**	3*	9.1	2.9	13.2*	1.6	30.1**	3.9	1.1	5.0		17.1**	9.1	3.2	11.4*	14**	34.7**
IBA+BAP 1.4+2.0	-32.5**	11.3*	-4.1	-4.3	-14.7**	-3.5	-18.9**	13*	-14.2**	-16**	-12**	-17.1**		-8.3	-13.7*	-5.7	-2.5	17.5**
IBA+BAP 1.4+2.5	-24.4**	18.4**	3.9	0.0	-5.2	4.1	-10.3*	21**	-6.2	-8.3	-4.1	-9.1	1.8		-5.9	2.3	5.9	25.5**
IBA+BAP 1.4+3.0	-18.5**	24.3**	8.8	5.9	-0.3	9.7	-5.3	25.3**	-3.3	-2.1	1.8	-3.2	13.7*	5.9		8.2	11.5*	31.5**
IBA+BAP 1.4+4.0	-25.8**	16**	1.6	-2.3	-6.5	1.6	-13.2*	18.7**	-6.5	-10.3*	-6.4	-11.4*	5.7	-2.3	-8.2		3.2	22.2**
IBA+BAP 1.5+5.0	-30**	12.8*	-1.7	-5.5	-11.8*	-1.5	-15.4**	15.5**	-11.9*	-13.5*	-9.7	-14.7**	2.5	-5.5	-11.5*	-3.2		20**
IBA+BAP 1.6+2.0	-50**	-7.2	-27.1**	-25.5**	-31.8**	-21.5**	-36.4**	-4.5	-31.3**	-31.5**	-29.7**	-34.7**	-17.5**	-25.5**	-31.5**	-22.2**	-20**	

* Indicates the combinations are showing significance at p<0.05 level.

** Indicates the combinations are showing significance at p<0.001 level

Appendix 2.b: ANOVA Table showing significance between the combinations on callus induction from leaf explants by *Post hoc* – tukey test

	Mix-1	Mix-2	Mix-NAA+B A-	BA+BAP +AS-	AS+BA- 20.0+0.2	BA+BAP 0.5+2.0	BA+BAP 1.0+3.0	BA+BAP 1.2+2.0	BA+BAP 0.5+2.0	BA+BAP 0.5+5.0	BA+BAP 0.5+1.0	BA+BAP 1.0+1.5	BA+BAP 0.5+2.0	BA+BAP 0.5+2.5	BA+BAP 0.5+5.0	BA+BAP 0.5+5.0	BA+BAP 0.5+1.0	BA+BAP 0.5+2.0
Mix-1		41.1**	24.9**	36.4**	21.5**	32.5**	26.2**	2.5	32.5**	55.4**	38.2**	53.2**	65**	30.5**	4.7**	56.2**	14.3**	56.9**
Mix-2	-41.1**		-16.2**	-11.7**	-19.5**	-8.5	-12.9*	-38.5**	-8.2	14.3**	-2.9	12.1*	23.9**	-4.6	-36.3**	15.4**	-26.8**	15.8**
BA+BA+BA-0.5+0	-24.9**	16.2**		5.5	-3.2	7.7	3.4	-22.4**	8.0	30.9**	13.4**	28.8**	40.7**	11.9*	-32.7**	31.5**	-11.5*	31.8**
BA+BAP+AS-0.2+	-36.4**	11.7*	-5.5		-8.5	2.2	-3.2	-47.3**	2.5	25**	7.8	22.8**	34.9**	5.1	-25.5**	25**	-16.1**	26.2**
AS+BA-20.0+0.2	-2.5**	19.5**	3.2	8.5		1.7*	5.5	-19.1**	11.2*	33.4**	15.8**	31.5**	43.4**	14.3**	-25.4**	34.0**	-7.3	33**
BA+BAP-0.5+2.0	-32.5**	8.5	-7.7	-2.2	-1.7*		-4.4	-38.1**	2.5	22.8**	9.5	20.5**	32.4**	3.9	-34.4**	23.3**	-18.3**	24**
BA+BAP-0.5+1.0	-26.2**	12.9*	-1.4	2.2	-4.5	4.4		-42.7**	4.7	27.2**	9.7*	25**	36.9**	8.3	-28.8**	28.2**	-13.9**	28.6**
BA+BAP-1.2+2.0	-2.5	38.5**	22.4**	27.3**	19.1**	30.1**	25.7**		33.4**	52.9**	35.7**	50.7**	62.5**	34**	-5.4**	54**	-11.1*	56.1**
BA+BAP-0.5+2.0	-32.5**	8.2	-4.3	-2.5	-11.2*	-1.3	-4.7	-38.4**		22.5**	9.3	20.3**	32.1**	3.6	-34.1**	23.5**	-18.5**	23.7**
BA+BAP-0.5+1.0	-55.4**	-14.3**	-30.9**	-25**	-33.8**	-22.8**	-27.2**	-45.3**	-22.5**		-17.2**	-2.2	9.8*	-18.9**	1.1	1.3	-41.1**	-1.2
BA+BAP-0.5+1.0	-38.2**	2.9	-13.4**	-7.8	-16.5**	-5.5	-1.0	-35.7**	-4.3	17.2**		15**	26.8**	-1.7	-18.8**	18.2**	-23.5**	18.6**
BA+BAP-0.5+1.5	-53.2**	-12.1*	-28.4**	-22.8**	-31.5**	-20.5**	-25**	-43.7**	-20.2**	2.2	-15**		11.8*	-16.7**	3.5	3.2	-38.9**	3.5
BA+BAP-0.5+2.0	-49**	-23.9**	-44.1**	-34.5**	-43.4**	-32.4**	-36.8**	-52.5**	-32.1**	-9.5*	-25.8**	-11.8*		-28.5**	4.1	4.5	-53.7**	-4.4
BA+BAP-0.5+2.5	-36.5**	4.5	-11.9*	-5.1	-14.3**	-3.3	-8.1	-34**	-3.5	18.9**	1.7	16.7**	28.5**		20.5**	20**	-22.2**	20.1**
BA+BAP-0.5+3.0	-5.7**	-15.9**	-32.2**	-26.5**	-35.5**	-24.4**	-28.8**	-54.5**	-24.1**	-1.1	-16.9**	-3.8	8.0	-20.5**		4.5	-42.7**	4.4
BA+BAP-0.5+4.0	-56.9**	-15.4**	-31.5**	-25**	-34.8**	-23.9**	-28.2**	-54**	-23.7**	-1.1	-16.2**	-3.2	8.5	-20**	0.5		-42.1**	0.2
BA+BAP-0.5+5.0	-14.3**	26.8**	10.9*	16.1**	7.3	18.3**	13.9**	-11.8*	18.5**	41.1**	23.9**	38.9**	50.7**	22.2**	-6.2**	42.1**		42.3**
BA+BAP-0.5+2.0	-56.9**	-15.5**	-31.8**	-26.2**	-35**	-2.4**	-28.4**	-54.1**	-23.7**	-1.2	-16.4**	-3.4	8.4	-20.1**	0.4	-3.2	-42.3**	

*Indicates the combinations are showing significance at p<0.05 level.

** Indicates the combinations are showing significance at p<0.001 level

Appendix 2.c: ANOVA Table showing significance between the combinations on percentage of callus induction from leaf disc culture by *Post hoc* – tukey test

	1.0+0.3	1.0+0.4	1.5+0.3	2.0+0.2	2.0+0.3	2.0+0.4	2.0+0.5	2.0+0.6	2.5+0.3	2.5+0.4	3.0+0.0	3.0+0.4	3.0+0.5	3.0+0.6	3.5+0.4	4.0+0.4
1.0+0.3		7.2	30**	30.6**	31.7**	32.9**	-12.6*	5.5	35**	35.5**	13.9**	-0.5	4.2	25.9**	22.2**	33.9**
1.0+0.4	-7.2		22.8**	23.4**	24.5**	25.7**	-20**	-1.7	27.9**	28.4**	6.7	-7.7	-3.4	18.4**	15**	26.7**
1.5+0.3	-30**	-22.8**		0.6	1.7	2.8	-43.8**	-24.5**	5.0	5.6	-15.1**	-30.5**	-26.2**	-4.4	-7.8	3.9
2.0+0.2	-30.6**	-23.4**	-0.6		1.1	2.2	-43.4**	-25**	4.4	5.0	-15.7**	-31.1**	-26.8**	-5.0	-8.3	3.3
2.0+0.3	-31.7**	-24.5**	-1.7	-1.1		1.1	-44.5**	-26.1**	3.3	3.9	-17.8**	-32.2**	-27.9**	-6.1	-9.4*	2.2
2.0+0.4	-32.9**	-25.7**	-2.8	-2.2	-1.1		-45.7**	-27.2**	2.2	2.8	-18.9**	-33.3**	-28**	-7.2	-10.5*	1.1
2.0+0.5	12.6*	20**	43.8**	43.4**	44.5**	45.7**		18.5**	47.9**	48.4**	26.7**	12.5*	17.8**	38.4**	35**	46.7**
2.0+0.6	-5.5	1.7	24.5**	25**	26.1**	27.2**	-18.5**		29.5**	30**	8.3	-6.1	-0.7	20**	16.7**	28.3**
2.5+0.3	-35**	-27.8**	-5.0	-4.4	-3.3	-2.2	-47.9**	-29.5**		0.6	-21.1**	-35.5**	-31.2**	-9.4*	-12.8*	-1.1
2.5+0.4	-35.5**	-28.4**	-5.6	-5.0	-3.9	-2.8	-48.4**	-30**	-0.6		-21.7**	-36.1**	-31.8**	-10*	-13.3**	-1.7
3.0+0.0	-13.9**	-6.7	16.1**	16.7**	17.8**	18.9**	-26.7**	-8.3	21.1**	21.7**		-14.4**	-14.1**	11.7*	8.4	20**
3.0+0.4	0.5	7.7	30.5**	31.1**	32.2**	33.3**	-12.3*	5.1	35.5**	36.1**	14.4**		6.3	26.1**	22.8**	34.4**
3.0+0.5	-6.2	1.4	23.2**	23.8**	24.9**	26**	-12.9*	-5.7	26.2**	26.8**	14.1**	-6.3		19.8**	16.4**	27.1**
3.0+0.6	-26.4**	-18.4**	-4.4	-5.0	-6.1	-7.2	-38.4**	-20**	9.4*	10*	-11.7*	-26.1**	-25.8**		-3.3	8.3
3.5+0.4	-22.2**	-15**	7.8	8.3	9.4*	10.5*	-35**	-16.7**	12.8*	13.3**	-8.4	-22.8**	-22.4**	3.3		11.6*
4.0+0.4	-33.9**	-26.7**	-3.9	-3.3	-2.2	-1.1	-46.7**	-28.3**	1.1	1.7	-20**	-34.4**	-34.1**	-8.3	-11.6*	

*Indicates the combinations are showing significance at p<0.05 level.

** Indicates the combinations are showing significance at p<0.001 level

Appendix 2.d: ANOVA Table showing significance between the combinations on percentage of bud induction from leaf disc culture by *Post hoc* – tukey test

	1.0+0.3	1.0+0.4	1.5+0.3	2.0+0.2	2.0+0.3	2.0+0.4	2.0+0.5	2.0+0.6	2.5+0.3	2.5+0.4	3.0+0.4	3.0+0.6	3.5+0.4	4.0+0.4
1.0+0.3		11.7	17.2*	13.9*	3.9	11.7	-14.5*	-7.8	16.1*	30**	13.9*	-5.1	-4.5	-8.9
1.0+0.4	-11.7		5.5	2.2	-7.8	0.0	-26.1**	-19.4**	4.4	18.3**	2.2	-17.8*	-16.1*	-20.6**
1.5+0.3	-17.2*	-5.5		-3.3	-13.3*	-5.5	-31.7**	-25**	-1.1	12.8*	-3.3	-23.3**	-21.7**	-26.1**
2.0+0.2	-13.9*	-2.2	3.3		-10.0	-2.2	-28.3**	-21.6**	2.2	16.1*	0.0	-20**	-18.3**	-22.8**
2.0+0.3	-3.9	7.8	13.3*	10.0		7.8	-18.3**	-11.6	12.2	26.1**	10.0	-10.0	-8.3	-12.8*
2.0+0.4	-11.7	0.0	5.5	2.2	-7.8		-26.1**	-19.4**	4.4	18.3**	2.2	-17.8*	-16.1*	-20.6**
2.0+0.5	14.5*	26.1**	31.7**	28.3**	18.3**	26.1**		6.7	30.5**	44.4**	28.3**	8.3	10.0	5.6
2.0+0.6	7.8	19.4**	25**	21.6**	11.6	19.4**	-6.7		23.9**	37.7**	21.6**	1.6	3.3	-1.1
2.5+0.3	-16.1*	-4.4	1.1	-2.2	-12.2	-4.4	-30.5**	-23.9**		13.9*	-2.2	-22.2**	-20.5**	-25**
2.5+0.4	-30**	-18.3**	-12.8*	-16.1*	-26.1**	-18.3**	-44.4**	-37.7**	-13.9*		-16.1*	-36.1**	-34.4**	-38.9**
3.0+0.4	-13.9*	-2.2	3.3	0.0	-10.0	-2.2	-28.3**	-21.6**	2.2	16.1*		-20**	-18.3**	-22.8**
3.0+0.6	5.1	17.8*	23.3**	20**	10.0	17.8*	-8.3	-1.6	22.2**	36.1**	20**		1.7	-2.8
3.5+0.4	4.5	16.1*	21.7**	18.3**	8.3	16.1*	-10.0	-3.3	20.5**	34.4**	18.3**	-1.7		-4.4
4.0+0.4	8.9	20.6**	26.1**	22.8**	12.8*	20.6**	-5.6	1.1	25**	38.9**	22.8**	2.8	4.4	

* Indicates the combinations are showing significance at p<0.05 level.

** Indicates the combinations are showing significance at p<0.001 level

Appendix 2.e: ANOVA Table showing significance between the combinations on number of shoot buds formation from leaf disc culture by *Post hoc* – tukey|test

	1.0+0.3	1.0+0.4	1.5+0.3	2.0+0.2	2.0+0.3	2.0+0.4	2.0+0.5	2.0+0.6	2.5+0.3	2.5+0.4	3.0+0.4	3.0+0.5	3.0+0.6	3.5+0.4	4.0+0.4
1.0+0.3		-10.1	46.7**	3.3	-21.7*	12.1	-5**	-43.7**	33**	56**	6.7	-33.7**	-27.7**	-30**	-37.3**
1.0+0.4	10.1		56.7**	13.3	-11.7	22.3*	-47**	-33.7**	43**	66**	16.7*	-23.7**	-27.7**	-20*	-27.3**
1.5+0.3	-46.7**	-56.7**		-43.3**	-71.3**	-37.3**	-106.7**	-913**	-16.7*	9.3	-43**	-80.3**	-87.3**	-79.7**	-87**
2.0+0.2	-3.3	-13.3	43.3**		-25**	9.0	-50.3**	-47**	23.7**	55.7**	3.3	-37**	-41**	-33.3**	-40.7**
2.0+0.3	21.7*	11.7	71.3**	25**		34**	-25.3**	-22*	54.7**	80.7**	28.3**	-12.0	-15*	-8.3	-15.7
2.0+0.4	-12.3	-22.3*	37.3**	-9.0	-34**		-59.3**	-56**	20.7*	46.7**	-5.7	-46**	-50**	-42.3**	-49.7**
2.0+0.5	5**	-4**	106.7**	50.3**	35.3**	59.3**		13.3	80**	116**	83.7**	23.3**	19.3*	27**	19.7*
2.0+0.6	43.7**	33.7**	913**	47**	22*	56**	-13.3		73.7**	102.7**	50.3**	10.0	6.0	13.7	6.3
2.5+0.3	-33**	-43**	16.7*	-20.7**	-54.7**	-20.7*	-80**	-73.7**		29**	-29.3**	-66.7**	-70.7**	-63**	-73.3**
2.5+0.4	-56**	-66**	-9.3	-56.7**	-80.7**	-45.7**	-116**	-102.7**	-29**		-52.3**	-82.7**	-86.7**	-89**	-96.3**
3.0+0.4	-6.7	-16.7*	43**	-3.3	-28.3**	5.7	-63.7**	-50.3**	29.3**	52.3**		-43.3**	-44.3**	-36.7**	-44**
3.0+0.5	33.7**	23.7**	83.3**	30**	12.3	46**	-23.3**	-10.0	96.7**	82.7**	43.3**		-4.0	3.7	-3.7
3.0+0.6	37.7**	27.7**	87.3**	41**	9**	50**	-19.3*	-6.0	70.7**	96.7**	44.3**	4.0		7.7	1.3
3.5+0.4	30**	20*	79.7**	33.3**	8.3	42.3**	-27**	-13.7	63**	89**	36.7**	-3.7	-7.7		-7.3
4.0+0.4	37.3**	27.3**	87**	40.7**	15.7	49.7**	-19.7*	-6.3	73.3**	95.3**	44**	3.7	-0.3	7.3	

* Indicates the combinations are showing significance at p<0.05 level

** Indicates the combinations are showing significance at p<0.001 level

Appendix 2.f: ANOVA Table showing significance between the combinations on number of shoot formation from leaf disc culture by *Post hoc* – tukey|test

	1.0 • 0.3	1.0 • 0.4	1.5 • 0.3	2.0 • 0.2	2.0 • 0.3	2.0 • 0.4	2.0 • 0.5	2.0 • 0.6	2.5 • 0.3	2.5 • 0.4	3.0 • 0.4	3.0 • 0.5	3.0 • 0.6	3.5 • 0.4	4.0 • 0.4
1.0 • 0.3		-7.7	35.3**	-9*	-4.0	9.3*	-45.7**	-4.0	35.7**	47**	13**	-6.3*	-10.3*	3.0	1.0
1.0 • 0.4	7.7		43**	-1.3	3.7	17**	-9*	3.7	43.3**	54.7**	20.7**	-0.7	-2.7	10.7*	8.7*
1.5 • 0.3	-35.3**	-43**		-44.3**	-39.3**	-26**	-51**	-35.3**	0.3	11.7**	-22.3**	-43.7**	-45.7**	-32.3**	-34.3**
2.0 • 0.2	9*	1.3	44.3**		5.0	18.3**	-6.7	5.0	44.7**	56**	22**	0.7	-1.3	12**	10*
2.0 • 0.3	4.0	-3.7	39.3**	-5.0		13.3**	-11.7**	3.0	39.7**	51**	17**	-4.3	-6.3	7.0	5.0
2.0 • 0.4	-9.3*	-17**	26**	-18.3**	-13.3**		-25**	-13.3**	26.3**	37.7**	3.7	-17.7**	-19.7**	-6.3	-8.3*
2.0 • 0.5	15.7**	8*	51**	6.7	11.7**	25**		11.7**	51.3**	62.7**	28.7**	7.3	5.3	18.7**	16.7**
2.0 • 0.6	4.0	-3.7	39.3**	-5.0	0.0	13.3**	-11.7**		39.7**	51**	17**	-4.3	-6.3	7.0	5.0
2.5 • 0.3	-35.7**	-43.3**	-0.3	-44.7**	-39.7**	-26.3**	-51.3**	-39.7**		11.3**	-22.7**	-44**	-46**	-32.7**	-34.7**
2.5 • 0.4	-47**	-54.7**	-11.7**	-56**	-51**	-37.7**	-62.7**	-51**	-11.3**		-34**	-55.3**	-57.3**	-44**	-46**
3.0 • 0.4	-13**	-20.7**	22.3**	-22**	-17**	-3.7	-28.7**	-17**	22.7**	34**		-21.3**	-23.3**	-10*	-12**
3.0 • 0.5	8.3*	0.7	43.7**	-0.7	4.3	17.7**	-7.3	4.3	44**	55.3**	21.3**		-2.0	11.3**	9.3*
3.0 • 0.6	10.3*	2.7	45.7**	1.3	6.3	19.7**	-5.3	6.3	46**	57.3**	23.3**	2.0		13.3**	11.3**
3.5 • 0.4	-3.0	-10.7*	32.3**	-12**	-7.0	6.3	-18.7**	-7.0	32.7**	44**	10*	-11.3**	-13.3**		-2.0
4.0 • 0.4	-1.0	-8.7*	34.3**	-10*	-5.0	8.3*	-16.7**	-5.0	34.7**	46**	12**	-9.3*	-11.3**	2.0	

* Indicates the combinations are showing significance at p<0.05 level.

** Indicates the combinations are showing significance at p<0.001 level

APPENDIX 3

Reagents for DNA isolation

3.a. DNA Extraction buffer

1M Tris HCl, pH 8.0

4M NaCl

0.5M EDTA

3.b. TE (Tris EDTA buffer, pH-8.0)

10mM Tris

1mM EDTA

3.c. 1X TAE buffer (pH-8.0)

40mM Tris Acetate

1mM EDTA

3.d. 6X Gel loading dye

0.03% Bromophenol Blue

0.03% Xylene Cyanol

60% Glycerol

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