Curcin isoforms in Jatropha (*Jatropha curcas* L.): sequence analysis, studies on amplicon profile and expression patterns in different tissues

A

Dissertation

Submitted in the partial fulfillment of the requirement for the award of degree of

Master of Science
In
Biotechnology

Submitted by:
Harsimrat Kaur
(Regd. No. 301401005)

Under the guidance of
Dr. N. Das
Professor

Department of Biotechnology
Thapar University, Patiala

July, 2016

CANDIDATE'S DECLARATION

I, hereby declare that the work which is being presented in the thesis entitled, **"Curcin isoforms in Jatropha (*Jatropha curcas* L.): sequence analysis, studies on amplicon profile and expression patterns in different tissues"** in the partial fulfillment of the requirement for the award of degree of Master of Technology in Biotechnology, Thapar University, Patiala, is an authentic record of my own research work carried out under the guidance and supervision of **Dr. N. Das**, Professor, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this dissertation has not been submitted to any other university or institute for award of any other degree.

Date: July 15, 2016
Place: Patiala

Harsimrat Kaur
(Harsimrat Kaur)

i

CERTIFICATE

This is to certify that the dissertation entitled **"Curcin isoforms in Jatropha (*Jatropha curcas* L.): sequence analysis, studies on amplicon profile and expression patterns in different tissues"** submitted by **Harsimrat Kaur** (Regd. No. 301401005) in partial fulfillment of the requirement for the award of the degree of Master of Technology in Biotechnology, to Thapar University is a record of student's own work carried out by her under our guidance and supervision. The report has not been submitted for the award of any other degree or certificate in this or any other university or institute.

15/05/2016

(Dr. N. Das)
Supervisor
Professor
Department of Biotechnology
Thapar University
Patiala

(Dr. Dinesh Goyal)
Professor and Head
Department of Biotechnology
Thapar University
Patiala

(Dr. S S Bhatia)
Dean of Academic Affairs
Thapar University
Patiala

ACKNOWLEDGEMENT

This is a golden opportunity for me to convey my sincere regards for all those people who enabled me to accomplish my dissertation work successfully.

My utmost gratitude goes to my esteemed advisor **Dr. Niranjan Das**, Professor Department of Biotechnology Thapar University, Patiala for his expertise guidance, kindness, motivation, patience and providing me the opportunity to complete my dissertation work. It is his confidence-imbibing attitude, splendid discussions and endless endeavors through which I have gained a lot and learned a lot building up my future and personality.

A special thanks to all faculty members for their constant encouragement and support throughout the project work.

I am thankful for the help rendered by both PhD throughout my project period without which it would have been difficult for me to achieve my goals. My special thanks to **Ms. Gurpreet Kaur** and **Mr. Rajneesh Verma**. .

I feel lacunae of words to express my most heartfelt and cordial thanks to my family and friends especially to **Preeti khokkar**, **Nitika Garg, Preetika, Shitanshu** and my entire class who always stood by my side during all the tough times.

I am thankful for the help rendered by lab attendents throughout my project period without which it would have been difficult for me to achieve my goals. My special thanks to **Mr. Ram Nawal Yadav**, **Mr. Lallan Yadav**, **Mr. Chandan Bhandari** and **Mr. Surinder kumar**.

The whole credit of my achievements during the project work goes to my parents and my guide. It was their unshakeable faith in me that has always helped me to proceed further.

Harsimrat Kaur

List of Contents

List of Tables

List of Figures

LIST OF ABBREVIATIONS

Abbreviations	Name
Bp	Base-pair
BSA	Bovine serum albumin
dNTP	2′-deoxynucleoside-5′-triphosphate
EDTA	Ethylenediamine-tetra acetic acid
IU/mL	International unit per mL
kb	Kilo base
kDa	Kilo-daltons
M	Molar
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide
mM	Milli-molar
nm	nanometer
N	Normal
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pmoles	picomoles
RIPs	Ribosome inactivating proteins
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TE	Tris EDTA
UTR	Untranslated region
µL	Microlitre
µg	Microgram
U/mL	Unit per mL

ABSTRACT

As of now, Jatropha (*Jatropha curcas* L.) stands as the best solution to the problems associated with production of biofuels, as it is well adapted to marginal soil with low nutrient content, and also suitable in different types of soil conditions, rainfall and climates. Disadvantage of *Jatropha* is the presence of toxic substances, particularly in the seeds, like curcin and phorbol esters which limits their applications. Curcin is the major toxin present in *Jatropha*. It is mainly found in the endosperm of the seeds of *Jatropha*. Considerable progress has been made on curcin as revealed in literature survey. Sequence analysis and comparison were made using the available curcin sequences. Apart from sequence relatedness, conserved amino acids were found and some important motifs were predicted which are presented in the comprehensive manner in this report. Hydropathy character of curcin was presented. Multiple sequence alignment was made using different forms of curcin namely curcin precursor, curcin2A, curcin-L precursor, ribosome inactivating protein (RIP) and ribosome-inactivating protein cucurmosin-like, *Jatropha* curcas. This exercise revealed both sequence identity and divergence between them. Good quality genomic DNA was isolated and purified from field-grown tender leaves using a simple and efficient protocol as introduced in our laboratory. PCR were carried out using curcin2A and curcin precursor cDNA-specific oligonucleotide primers. The amplicons corresponding to the individual PCR were noted in order to analyse and compare between the *Jatropha* accessions. RNA was isolated and purified from different tissues of plant using a simple and efficient protocol as introduced in our laboratory. RT-PCR approach was adopted to see the expression patterns of curcin2A and curcin precursor in different tissues of *Jatropha*. This study would be useful for further research on curcin gene in *Jatropha*.

CHAPTER-1 INTRODUCTION

1.1 The biodiesel crop *Jatropha* (*Jatropha curcas* L.)

Jatropha curcas L. (*Euphorbiaceae*) or purging-nut or physic nut is an all-purpose, zero waste perpetual plant. The plant is drought repellent, multipurpose crop and is gaining a lot of importance for biodiesel production. *Jatropha* species cultivated in India are *Jatropha curcas, Jatropha integerrima, Jatropha glandulifera, Jatropha nana, Jatropha gossypifolia, Jatropha multifida*. The oil produced by this crop, can meet the American and European standards as it can be easily transformed into liquid bio-fuel. The crops are highly toxic and cannot be used for cattle feed. *Jatropha* occurs mainly with annual temperatures above 20°C at lower altitudes, but can grow at higher altitudes and can bear little frost also.

Taxonomy of Jatropha curcas: The genus *Jatropha* belongs to tribe *Joannesieae* in the *Euphorbiaceae* family and contains approximately 175 known species. Linnaeus (1753) named the physic nut *Jatropha* L. in "Species Plantarum", and is still valid to-date. The genus name *Jatropha is* derived from the Greek word *jatr ´os* (doctor) and *troph´e* (food), which implies medicinal uses.

Taxonomic hierarchy of *Jatropha curcas*

Kingdom- *Plantae*
Division- *Magnoliophyta*
Class- *Magnoliopsida*
Order- *Malpighiales*
Family- *Euphorbiaceae*
Subfamily- *Crotonoideae*
Tribe- *Joannesieae*
Genus- *Jatropha*
Species- *curcas*

Botanical features of Jatropha: The physic nut by definition is diploid, deciduous large shrub or small woody tree of 3 to 5 m in height but under favourable conditions it can attain a height of 8 or 10 m. The plant shows flexible growth with a structural discontinuity at each increment. It has a grey bark which when cut secretes white latex. Normally, from seedlings, five roots are formed, one central and four peripheral. A tap root is not usually formed by vegetative propagated plants. Under normal conditions it fruits annually. More than 1.5 to 2.0 kg seeds

could be produced per plant per year. The plant could survive for about 40-50 years and it is well adapted to semi-arid climates. The trees are deciduous and shed the leaves in dry season.

Fig. 1 Leaf, flower, fruits and seeds of *Jatropha* (Ref: www.plantattraction.com)

Leaf of this plant is large (10-15cm) in length, 4-6 lobed and is heart shaped. They are initially yellowish green in colour and at maturity they become dark green. A flower is formed terminally, discretely, with female flowers (tricarpellary, syncarpous with trilocular ovary) usually slightly larger and occurs in the hot seasons during July to September.

Fruits are 3 cm long and generally tri-halved and grey-brown capsule in shape. Each fruit consists of three seeds. Each bloom yields a bunch of approximately 10 or more ovoid fruits. Maturation of fruits takes place from October-November depending upon the climatic conditions and best time to harvest the fruits is when the colour changes from yellow to dark brown and the seeds mature by three months after flowering.

Seeds are usually harvested after one year of plantation. The seeds mature about 3–4 months after flowering. The seeds are black in colour and the seed weight per 1000 is about 727 g, there are 1375 seeds/kg in the average. Oil content in the seed is around 25-40 % by weight of this plant. Seeds contain about 22 % saturated fatty acids and 78 % unsaturated fatty acids. In *Jatropha* oil oleic and linoleic acids are mainly present (Rehm *et al.*1991). The seeds also consist of some other chemical compounds, such as, glucose, fructose, galactose, saccharose, raffinose and toxic protein like curcin.

Distribution and cultivation of Jatropha: The Portuguese seafarers distributed *Jatropha* via the Cape Verde Islands and Guinea Bissau to other countries in Asia and Africa (Heller, 1996). The plant is well adapted to all the conditions (arid and semi arid). *Jatropha* is cultivated in many

states of India which are Gujarat, Madhya Pradesh, Rajasthan, Maharashtra and Tamil Nadu. Cultivation of *Jatropha* is done by two methods either by vegetative propagation (cuttings) or through generative propagation (seeds). Propagation of plant through cuttings is slower than a seed i.e. through seeds propagation is fast. Another factor in favour of productivity is the ratio of female and male flowers, more the number of female flowers results in more number of fruits. Seed bearing in *Jatropha* starts within first 9-12 months of its plantation but best seed yield is observed after 2-3 years of its plantation. Not all factors are of equal importance.

Uses of *Jatropha* products:

- *As green manure and fertilizers*: Seed cake is a by-product of oil extraction also called press cake. *Jatropha* seed cake contains a highly toxic protein called curcin which is similar to ricin in castor, which makes it unsuitable for cattle feed. However, it has a potential as fertilizer or for biogas production (Staubmann *et al.*, 1997; Gubitz *et al.*, 1999). The seed cake is an exceptional source of plant nutrients as it is abundant in nitrogen. *Jatropha* seed cake is also used as a straight fertilizer, with regard to nitrogen, phosphorus and potassium content, its properties were compared which is shown in the Table below:

Table 1 Nutritional analysis of oil seed cakes and manure (Delgado and Parado 1989)

Property	*J. Curcas* oil cake	Neem oil cake	Cow dung manure
Nitrogen	3.2–4.44	5.0	0.97
Phosphorus	1.4–2.09	1.0	0.69
Potassium	1.2–1.68	1.5	1.66

Jatropha seed cake could be a good source of low cost production of industrial enzyme showed a recent experiment on solid state fermentation (Mahanta *et al.*, 2008).

- *As food: Jatropha* can be toxic when consumed, however, in some parts of Mexico and Central America, reportedly a non-toxic variety of *Jatropha* exists which is said that it does not contain toxic Phorbol esters (Makkar *et al.*, 1998). The young leaves may be eaten safely, "steamed or stewed" and after roasting the seeds the variety is used for human consumption. (Duke, 1985a; Ochse, 1931). Levingston and Zamora (1983) reported that once the embryo has been removed, the seeds are edible. The seeds of Mexican origin have less toxic contents so that the seeds can be eaten with proper processing.
- *Industrial Uses:* A by-product of biodiesel that is glycerin can be used to make soap, and it can be produced from *Jatropha* oil itself. In both cases the process produces a

soft, durable soap which is well adapted to household or small-scale industrial activity. Dye is used for dying cloth which is obtained from bark and leaves.

- *Insecticidal property:* The aqueous extract from oil has potential as an insecticide. It has been used in the control pests of pulses, potato and corn and insect pests of cotton including cotton bollworm (Kaushik and Kumar, 2004). Biodegradable toxins present in methanol extracts of *Jatropha* seed are being tested in Germany for control of bilharzias carrying water snails.
- *Medicinal Uses***:** All parts of *Jatropha* i.e. seeds, leaves and bark have been used in traditional medicine and for veterinary purposes for a long time (Dalziel, 1955; Duke, 1985b; Duke, 1988). Uses of various parts of *Jatropha* have been presented in Table below:

Table 2 Uses of different parts of *J. Curcas* in medicines (Heller 1996, kaushik and kumar 2004)

Plant part	Diseases
Seeds	To treat gout, arthritis and jaundice
Stem	Toothache, gum bleeding, pyorrhoea
Plant sap	Dermatomucosal diseases
Water extract of branches	HIV, Tumor
Plant extract	Wound healing, Allergies, burns

Biodiesel from physic nut: Biodiesel is made from both edible and non-edible oil and animal fats through transesterification and is a substitute for diesel and requires very little engine modifications up to 20% blend and minor modification for higher percentage blends. In diesel engines *Jatropha* oil can be used as fuel directly or it can be blended with methanol (Gubitz *et al.,* 1999). In *Jatropha* seeds oil content is in high amount ranging from 30-40 %. Biodiesel usually contains long chain alkyl-esters. Biodiesel does not produce Sulphur and Carbon monoxide (CO) on burning therefore it is biodegradable, non-polluting and environment friendly in nature. As compared to commercial diesel, it has lesser mutagenic potency. Greenhouse gas emissions are reduced up to 50 % (Mukharjee *et al.* 2011).

1.2 Toxic compounds in *Jatropha*

Jatropha contains many toxic compounds such as lectins (curcin), saponins, phorbol esters and protease inhibitors. All these toxic substances are found in seeds of *Jatropha* (Sujatha 2009)**.** *Jatropha* oil contains approximately 30% phorbol esters (Devappa 2010). Due to all these toxic substances found in *Jatropha curcas*, it cannot be used for cattle as a fodder and as cooking oil for animals. Adams in 1975 studied that when two goats were given *Jatropha* seeds 1g/day,

they died within 2 to 9 days while on decreasing the amount of *Jatropha* seeds i.e. 0.25g/day and 0.5g/day, they both died within 11 to 21 days. This study revealed that seeds, press cake and oil of *Jatropha* cannot be utilized for human or animal feed.

1.3 Curcins: toxic proteins in *Jatropha*

Toxicity of seeds is due to the protein component. This protein component was first extracted from the seeds of *Jatropha* by Felke (1914), and he named this protein as "Curcin. It is mainly found in the endosperm of the seeds of *Jatropha* (Mourgue *et al.*1961). Felke in 1914 proposed that the curcin was a kind of toxalbumin. Barbieri et al. (1993) described that curcin is a type 1 RIP. Curcin is a highly toxic RIP similar to ricin, the phytotoxin found in the castor bean (Ricinis). With the help of protein-ligand docking approach the r-RNA binding site of curcin can be identified. Cloning of a peptide inhibitor to the r-RNA binding site of curcin can produce non-toxic varieties of *Jatropha curcas*. Similar to a series of the pokeweed antiviral protein phytotoxins, the curcin 2, curcin 3, and curcin 4 have approximate half maximal inhibitory concentration (IC50) in the cell-free system and a slightly lower IC50 in the whole cells system (Barbieri *et al*. 1993).

1.4 Medicinal potential of Curcin

Crude curcin can be used as component of 'Immunotoxins' which is a hybrid molecule consisting of toxic peptide linked to an antibody (Frankel *et al.* 1986). Crude curcin have poor stability and immunogenicity so it cannot be used as components of Immunotoxins (Kreitman *et al.* 1999). Oil from *Jatropha curcas* seeds is used to cure skin ailments and in treatment of rheumatism (Heller 1996).

Anthelmintic Activity of Curcin: Although the majority of Helminth infections in humans are restricted to tropical regions only but it is worrying issue in today's world. Helminth infection leads to various healths hazardous problems such as anaemia, eosinophilila and pneumonia (Bundy 1994). Jummai *et al.* in 2014 studied medicinal potentials of *J.curcas* on Helminth infection. They used Niclosamide as reference standard and normal saline as the control in the experiment. The result of the experiment shows that the crude curcin was more potent on Helminth infection than the standard (Niclosamide). *Jatropha curcas* consist of alkaloids and saponins in a huge amount (Ahirrao *et al*. 2011), which stop the supplies of glucose to helminthes which acts on CNS causing paralysis (Mute 2009; Sutar *et al.* 2010; Sharma *et al.* 2010; Mali *et al.* 2007).

Antitumor activity of Curcin: It was observed that curcin protein could inhibit the growth of tumor cells at very low concentration. Various observations proposed that RIPs can kill the tumor cells by apoptosis (Luo *et al.* 2007). The anti-tumor activity of curcin was checked by

Luo *et al.* in 2007 on cellule pulmonary cancer cells (NCL-H446) and gastric cancer cells (SGC-7901).They inserted the fragment of DNA encoding the mature protein of curcin *E.coli* strain M15 and used 0.5mM isopropyl- β-D-thiogalactopyranoside as inducer. The recombinant protein was expressed in the form of the inclusion body. They purified this recombinant protein by Ni-NTA affinity chromatography and used different concentration of this purified protein to kill the tumor cells for different time. They observed that curcin protein inhibit the growth of cancer cells at a very low concentration. Juan et al. in 2003 studied the anti-tumor activity of curcin by MTT assay (3-(4, 5-dimethylthiazol-2)-2, 5 diphenyltetrazoliumbromide) which includes testing the toxic effects of curcin on the viability of cells grown in culture medium via counting the number of viable cells and their proliferation. The reduction of Tetrazolium salts was used to check the cell proliferation. They observed that curcin have anti-tumor activity and the mechanism behind anti-tumor activity was N-glycosidase activity. They observed that curcin didn't have harmful effects on normal cells. The MTT assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS) (Mosmann 1983). MTT assay are the colorimetric assay in which the yellow colour Tetrazolium MTT is reduced to purple colour formazan by metabolically active cells. The MTT Reagent yields low absorbance values in the absence of cells. During the last more than one decade considerable progress has been made on curcin research at cellular, biochemical, molecular and genetic levels which are described precisely in the following section.

CHAPTER- 2 REVIEW OF LITERATURE & OBJECTIVES OF THE STUDY

2.1 Chemical constituents of *Jatropha* seeds

A variety of sources are available on the fatty acid composition of *Jatropha* oil developing from different countries. It possesses the four foremost fatty acids namely, palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic acid (C18:2) (Rehm and Espig 1991). *J. curcas* seeds are composed of about 47% oil, 25% protein, and 10% fiber (Abulude, *et al* 2007).The chemical anatomy and insecticidal flurry of *Jatropha curcas* L. seed were explored by Adebowal and Adedire (2006) and they evaluated high oil content of the seed (66.4%). The dominant lipid species was Triacylglycenol, while1, 2-Dioleoyl-3-linoleoyl-rac-glycerol was the major triacyglycerol. Linolenic acid was the dominant fatty acid in the oil. Rug and Ruppel (2000) analysed that oil content is 25–30% in the seeds of *Jatropha curcas*. The oil holds saturated fatty acids (21%) and unsaturated fatty acids (79%). Matsuse, *et al* (1999) studied the aqueous and methanolic extracts of 39 Panamanian medicinal plants for anti-human immunodeficiency virus (HIV) effects. The water extract of the branches of *Jatropha curcas* reserved strongly the HIV-induced cytopathic effects with low cytotoxicity.

2.2 Medicinal Potentials of various parts of *Jatropha* plant

Leaves: The leaves and other parts of the plant are used for the treatment of various diseases. *J. curcas* leaves were believed to contain steroid sapogenins, alkaloids, the triterpenae alcohol, 1-triacontanol and a dimer of a tripene alcohol. Neuwinger, 1994; Staubmann *et al*., 1999 had isolated a complex of 5 hydroxypyrrolidin-2-one and pyrimidine-2, 4-dione from the leaves of *J. curcas* by extraction with ethyl acetate. In Oceania, on the island of Tonga, the leaves of *J. curcas* have been used in folk medicine to treat vaginal bleeding (Singh *et al.,* 1984). In Cape Verde and Cameroon, a decoction is used internally and externally against fever. In Cameroon, the leaves are also in use as the remedy against rheumatism and in Nigeria against jaundice (Staubmann *et al.,* 1999). The leaves are used extensively in West Africa for ethno medical practice in various forms to heal various ailments like fever, mouth infections, jaundice and joint rheumatism (Irvine, 1961; Oliver-Bever, 1986).

Stem bark, branches and twigs: Phytochemical shielding of *J. curcas* stem bark extracts disclosed the presence of secondary metabolites such as saponins, steroids, tannins, glycosides, alkaloids, flavonoids and also yields dark blue dye (Igbinosa *et al.,* 2009). Shimada (2006) investigated that tannins have been found to form compounds with proline rich protein

resulting in the hindrance of cell protein synthesis. Parekh and Chanda (2007) described that tannins reacts with protein to provide the typical tanning effect, vital for the treatment of inflamed or ulcerated tissues. Alkaloid is another secondary metabolite compound observed in the stem bark extract of *J.* Curcas. Alkaloids are the largest groups of phytochemicals in plants having surprising effects on humans, which has led to the evolution of potent painkiller medications (Kam and Liew 2002). Quinlan *et al.* (2000) handled steroidal extracts from few remedial plants, which showed antibacterial activities on some bacterial isolates. Neumann *et al.* (2004) also confirmed the antiviral property of steroids. Flavonoid is another secondary metabolic compound of *J. curcas* stem bark extracts. It revealed a broad range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties (Hodek *et al.,* 2002).

Latex: Folklore uses of *J. curcas* latex are to cure toothache, as a mouth rinse to treat bleeding gums, as a haemostatic, wound dressing and many others (Fazwishni and Kristiani, 2007). The latex contains tannin, saponin, waxand resin (Perry and Metzger, 1980; Watt and Breyer-Brandwijk, 1932). Fazwishni and Kristiani (2007) conducted mutagenicity test to evaluate the mutagenicity of the latex by Ames method and the result showed that *J. curcas* latex produces no mutagenicity activity. The latex can be used as anti inflammatory by massaging it to the traumatic area (De Feo, 1989).

Fruits and seeds: The seeds of *J. curcas* have good potentials as a fuel substitution. However, the seeds in general, are toxic to human and animals. A toxic protein (curcin) which is extracted from seeds also contains a high concentration of phorbol esters (Adolf *et al.,* 1984; Makkar *et al.,* 1997). Two new esterases (JEA and JEB) and a lipase (JL) were isolated from the seeds (Staubmann *et al.,* 1999). Esterases have been isolated from mammalian tissue as well as from microorganisms and plants because of the simple availability; microbial esterases are of interest for application in industrial processes. Lipases constitute a significant portion of enzymes that have been investigated for use in organic synthesis (Hills *et al.,* 1990). Lipases have possible use in lipid refinement to produce synthetic lipids for many industrial uses (Macrae, 1983; Svensson *et al.,* 1992). In Egypt, the seeds are used for the treatment of arthritis, gout and jaundice (Khafagy *et al.,* 1977). The seed oil can be applied to treat eczema and skin diseases and to sooth rheumatic pain (Heller, 1996). The oil is also used externally for the treatment of sciatica, dropsy and paralysis (Mujamdar *et al.,* 2000). The 36% linoleic acid (C18:2) content in *J. curcas* kernel oil is the possible interest for skin care (Kumar and Sharma, 2008).

Roots: J. curcas roots are used for treating eczema, scabies, ringworm and gonorrhea where these diseases are caused by fungi and bacterial infections (Aiyelaagbe *et al*., 2007). Chen *et al*. (1988) had isolated diterpenoids jatropholone A and B and a new diterpenoid named jatropholol from the roots of *J. curcas*. Previous work (Dekker *et al.,* 1986; Aiyelaagbe *et al.,* 2000; Aiyelaagbe, 2001) has shown that many roots of *Jatropha* species possess antimicrobial activities. The roots are also reported to be used as an antidote for snake bites. The roots of this plant are applied locally in paste form after crushing, for the treatment of inflammation by Bhil tribes from Rajasthan area in India on empirical basis (Joshi, 1995). The roots can also be used as a mouthwash for bleeding gums and toothache.

2.3 Genetic Diversity and Phylogenetics of *Jatropha* Species

Genetic diversity is one of the most valuable assets of the plant genetic resources. An effective way of exploiting and managing available genetic resources efficiently is characterisation of germplasm. Phlyogenetics of species is important to determine genetic relatedness within the species and among the species. Finite studies have been carried out on the genetic diversity and phlyogenetics of genus *Jatropha*. Puangpaka and Thaya (2003) studied the karyology of five *Jatropha* species by staining chromosomes of microsporocyte and reported that in most species, the chromosomes are coupled as bivalents at first metaphase and divided to 11:11 at first anaphase. Based on cytological and peroxidase isozyme studies, Prabakaran and Sujatha (1999) reported *J.tanjorensis* as a natural interspecific hybrid of *J. Curcas* and *J.gossypifolia.* Genetic diversity and phylogenetic analysis using RAPD (Random Amplification of Polymorphic DNA), AFLP (Amplified fragment length polymorphism) and nrDNA ITS analysis suggested interspecific genetic divergence of 97.74% (RAPD), 97.25% (AFLP) and 0.419 by nrDNA ITS sequence (Sudheer *et al.* 2009a, 2009c). Phylogenetic trees constructed using RAPD, AFLP and nrDNA ITS sequence data supported the highest genetic similarity between *J. curcas* and *J. integ*errima.

2.4 Tissue Culture and Genetic Transformation

During the last few decades, studies on tissue culture and micropropagation have increased tremendously due to their wide utility in large scale multiplication and in genetic transformation. Winton (1968) regenerated the first complete plant from tissue culture of a tree species, since then protocols for micropropagation for many plants species have been established and exploited commercially. To meet the large scale demand and ensure a constant supply of quality planning material required in the development of mass multiplication

techniques. The inceptive steps in these approaches, i.e. micropropagation, plant regeneration and transformation, in improvising *Jatropha* spp. have been discussed (Sujatha *et al.* 2008).

2.5 *In Vitro* Micropropagation of *Jatropha*

Many efforts have been made to establish procedures for micropropagation and regeneration from different explants of *Jatropha*. From all parts of the plant i.e. hypocotyls, leaf, cotyledon, petiole and stem, induction of callus have been achieved (Sujatha *et al.* 2008; Rajore and Batra 2007; Kumar *et al.* 2008). Morphogenesis and regeneration (Sujatha and Mukta 1996; Shrivastava and Banerjee 2008), somatic embryogenesis (Sardana *et al.* 2000; Jha *et al.* 2007), regeneration from epicotyls callus (Qin *et al.* 2004; Rajore and Batra 2007), multiple shoot proliferation from shoot tips (Rajore and Batra 2005; Datta *et al.* 2007), and shoot bud proliferation axillary nodes and leaf sections of non toxic *J. curcas* have been reported (Sujatha *et al.* 2005). According to Kumar (2008), regeneration efficiency varies significantly according to source and type of explants. Regeneration in *J. curcas* is also reported to be highly genotype dependent (da Camara Machado *et al.* 1997).

2.6 Toxicity studies on *Jatropha*

Felke (1913) was the one who first isolated curcin. Later Stripe, *et al.* 1976 found that curcin hinders protein synthesis *in vitro*. Diterpenes have been isolated from seeds (Adolf, *et al.* 1984) and roots (Naengchomnong, *et al.* 1986; Chen, *et al.* 1988). In a mouse cocarcinogenesis skin tumour was promoted by these toxic substances. An epidemiological study is to be carried out on human cancer, it was suggested by Horiuchi *et al.* (1987). Wink (1993) reported that approximately 11% oil content is found in seed cake, in which the thermostable toxic diterpenes are bound. Aregheore, *et al* (1998) worked on the lectin activity using a latex agglutination method in toxic and non-toxic varieties of *Jatropha curcas* seed meal which suggested that in *J. curcas* meal: i) In inactivating lectins moist heat treatment is more effective than dry heat treatment ii) lectins can be completely inactivated using moist heat treatment at 121°C for 30 min, and iii) In *Jatropha* meal lectins probably are not the toxic principle. Gross, *et al* (1997) and Mittelbach *et al* (2000) attempted detoxification treatments on laboratory scale by press cake and oil to remove phorbolic esters and curcin followed by feeding experiments on fish and mice. The acute toxicity of *J.curcas* seeds was studied by Abdu-Aguye, *et al* (1986) and he revealed that when the seeds of *Jatropha curcas* L. were consumed accidentally by two children aged 3 and 5 years led to a clinical infirmity of severe vomiting, restlessness and dehydration.

2.7 Toxins in different parts of *Jatropha*

All *Jatropha* parts are toxic in nature, especially the seeds of plant. Lectin (curcin), phorbol esters and saponins are toxic substances in *Jatropha curcas* due to which seeds, press cake and oil cannot be used for human or animal feed (Sujatha 2009). In *Jatropha curcas* meal studies shows that lectin is not the higher toxicant component (Aderibigbe *et al.* 1997; Aregheore *et al.* 1998)

Lectins: Lectins are basically the proteins capable of binding with high affinity carbohydrate moiety (Goldstein and Hayes 1978). They play a major role against pathogen in plant defence (Chrispeels and Raikhel 1991). When dry seeds absorb water, lectins are released into the water (Fountain *et al.* 1977). This leads to the interaction of lectin with potential pathogen in germinating seed. Second method of lectin defence system is when pathogen eat the seeds of plant, lectin present inside the seeds is released and binds with the glycoproteins (present in the intestinal tract of pathogen) and absorption of nutrients is inhibited which led to death of pathogen. Lectin and trypsin inhibitors in *Jatropha* can be reduced by heat treatment (Aderibigbe *et al.* 1997).

Phorbol esters: Phorbol esters are found in both toxic and non-toxic varieties therefore this compound need to be studied further (Wink *et al*. 1997). Six different types of phorbol esters are found in the kernels of *Jatropha* seeds (Haas *et al.* 2002).In many plants of family Euphorbiacaeae phorbol esters are said to be found (Ito *et al*. 1983). With regards to their biological activities they are structure specific. Tumor is induced by Phorbol esters by activating the protein kinase and important cellular target proteins. Phorbol esters have been extracted and studied in various human and animal cell lines for the tumor inducing property (Goel *et al.* 2007). On guinea pigs toxic effects of phorbol esters was studied and positive results were found (Goel *et al.* 2007). Phorbol esters showed bio pesticides property against fresh water snails and it was highly toxic to silkworm larvae (Goel *et al.* 2007). Toxic fraction of *Jatropha* oil was extracted and applied on the skin of rabbits and rats which showed irritant reaction followed by the necrosis (Gandhi *et al*.1995). Phorbol esters which are main toxic agents in *Jatropha* are not inactivated by heat treatment (Aregheore *et al.* 1998). During the aerobic composting process phorbol esters can be detoxified by biochemical degradation process. Toxicity of the seed cake exposed to air decreased the amount of phorbol esters after 30 days (Devappa 2010). In Mexico a non-toxic variety of *Jatropha curcas* has been found, which after roasting can be consumable for human. By using fish and rats as experimental models, non-poisonous nature of this variety was checked. Some molecular markers are used to differentiate between toxic varieties of *Jatropha* and non toxic varieties. These Mexican

varieties (non-poisonous) of *Jatropha* have very low levels or nil levels of phorbol esters 0.27 mg g^{-1} in kernel meal as compared to the toxic varieties containing around 2.49 mg g^{-1} phorbol esters (Makkar *et al.*1997). But these non-toxic varieties still contain half the amount of toxic protein curcin as that of the toxic varieties, 30 % more trypsin inhibitors and 45-50% more saponins (Sujatha 2009).

Ribosome Inactivating Proteins (RIPs): Generally growth and yield of *Jatropha* plant is affected by various abiotic and biotic stresses and undergoes a number of physiological and developmental changes during cold and drought stress (Kazuo *et al*. 1996). Plants have developed complex defence mechanism against pathogen attacks (Bowles 1990). The defence mechanism evolved in plants includes Ribosome inactivating proteins (RIPs).Many plants contain proteins that are capable of inactivating ribosome and accordingly are called ribosome-inactivating protein (RIP). On the basis of their structure and functions, RIP are usually divided in 2 subgroups: Type I RIP consisting of a single polypeptide chain (*M*r 28 000-35 000) and alkaline isoelectric points (pI) of pH 8 to 10 with or without carbohydrates; type II RIP consisting of a catalytically active A chain linked to a cell-binding B chain. The A chain is the functional equivalent of a Type I RIP, and the B chain is a lectin (Barbieri *et al*., 1993). curcin is type I ribosome inactivating protein (Barbieri *et al.*, 1993). Curcina purgative oil and a phytotoxin or toxalbumin (curcin) is similar to ricin in Ricinis. In eukaryotic cells ribosome-inactivating proteins (RIPs) from plant are toxins that can inhibit protein synthesis by catalytically damaging ribosomes (Barbieri *et al.*1993). With rRNA N-glycoside activity, when entering a cell the protein can enzymatically cleave the specific glycosidic bond of an adenine A4234 of 28S rRNA, thus inhibiting protein synthesis by interfering with the binding of the 28SRNA to elongation factors (Peumnas *et al.*,2001). RIPs are involved in plant defence systems and they posses anti-fungal and anti-viral activities (Krawetz and Boston, 2000) as well as anti-tumour activity (Lin *et al.*, 2003). In addition RIPs are of great interest in theory and practice (Shaw *et al*., 1991). They have been used as a mid-term aborting agent (Cheung *et al.*, 1989) and in the treatment of hydration moles (Lu and Jin, 1990).

Curcin: The protein component is the cause of toxicity of *Jatropha* seeds. Felke (1914) extracted this protein component from the seeds of *Jatropha* and he named this protein as "Curcin" and proposed it to be kind of toxalbumin. It is mainly found in the endosperm of the seeds of *Jatropha* (Mourgue *et al*.1961). Barbieri *et al.* (1993) described that curcin is a type1 RIP. Cloning and characterization of Curcin, a toxin extracted from *Jatropha curcas* has revealed to encode the type-1 ribosome inactivating proteins (RIPs). Different forms of curcin gene are reported in NCBI database i.e. Curcin2A gene (Accession no. GQ925453), Curcin-L

precursor (Accession no. EU195892), Curcin gene (Accession no. EU395775), RIP partial *Jatropha curcas* gene (Accession no. AY435214) and Curcin precursor gene (Accession no. AF469003). Considerable differences could be noted between them at both nucleotide and amino acid sequence levels. After extraction of oil from *Jatropha* seeds, the seed meal contains high amount of curcin (Stripe *et al.* 1976). It is highly toxic RIP similar to ricin, the phytotoxin found in the castor bean (Ricinis). Amino acid sequence of coding region of Curcin gene shows high homology with other RIPs such as ricin A-chain, gelonin and momorcharin (Juan *et al.* 2003). Comparison between amino acid sequences of curcin and ricin-A chain shows high similarities with 54 % homology. The minimum lethal dose of ricin is very small as 0.0000001% of body weight when administered by injection (Kingsbury 1964). It can lead to disease such as dehydration and cardiovascular collapse as a result of haemorrhagic gastroenteritis, central nervous system depression. Although curcin was 1000 times less toxic than ricin (Juan *et al* 2003). With the help of protein-ligand docking approach the r-RNA binding site of curcin can be identified. Cloning of a peptide inhibitor to the r-RNA binding site of curcin can produce non-toxic varieties of *Jatropha curcas*.

2.8 Chemical structure and stability of Curcin

Curcin is a large complex phytotoxin type protein molecule with high toxicity. Phytotoxins are heat labile and easily identified by precipitin reaction with known antibodies (Kingsbury 1964). They match bacterial toxins in structure and in physiological effects. Curcin remains in the seed after the oil has been extracted. It is unable to penetrate cell walls as it lacks a carrier moiety or a galactose binding group which binds to cell membrane like that of ricin. This was observed in a cell free system, the activity of curcin was not enhanced by treatment with 2-mercaptoethanol while in ricin the inhibitory effect is increased by treatment with 2-mercaptoethanol as it splits the molecules of ricin into two parts, one is effector and another is carrier moiety (Stripe *et al*. 1976)

Extraction and purification of curcin: Crude curcin can be extracted from seeds of *Jatropha curcas* by solvent extraction method using hexane as a solvent and Phosphate buffer saline as an extracting buffer (Jummai *et al.* 2014).The crude curcin can be purified by using CM-52 column and Sephadex G-100(Juan *et al.* 2003).

2.9 Curcin cDNA sequences, and expression patterns of curcin in *Jatropha*

Juan *et al*. in 2003 studied the full length curcin cDNA sequence and its amino acid sequence which was obtained by the study of gene sequence (Genbank ID: AY069946).They found that first an immature protein with 293 amino acid was translated from amino acid sequence of curcin cDNA then after it's processing, a mature protein with 251 amino acids residues was

obtained. They revealed that Curcin contained one cysteine residues at 209th amino acid. Thus, it directly forms a disulphide linkage with an activated antibody. Therefore it can be used in the preparation of immunoconjugates having great potential as chemotherapeutic agents for the cure of cancer or AIDS (Juan *et al*. 2003)**.**

Expression of Curcin2 gene in Jatropha curcas under stress conditions: 'Inducible expression of curcin2 gene under drought, temperature stress and fungal infection was studied by Qin *et al*. in 2005. They used one month old seedlings of *Jatropha curcas* and treated with various concentration of PEG solution. After growing seedlings at 25°C, they were transplanted to different temperature conditions maintained at 4° to 50°C. After temperature treatment, they inoculated leaves of one-month seedlings with fungus. Results of this experiment showed that the accumulation of curcin2 gene was expressed specifically in leaves of seedlings growing under stress conditions like drought, temperature stress and fungal infection and not expressed in normal conditions i.e. non-stressed conditions (Qin *et al.* 2005). Either in stress or non-stress conditions, curcin gene is not expressed in leaves. Curcin gene expresses only in seeds and its molecular weight is 28.2 kDa (Lin *et al.* 2002), while curcin2 gene can expresses in leaves of seedlings growing under stress conditions. Molecular weight of curcin2 is 30.1 kDa (Qin *et al.* 2005)

2.10 Origin of the problem

Jatropha curcas has been a source of attraction to researchers across the world because of its potential use as biodiesel, a renewable energy source. But in developing countries like India these edible oils cannot be used for biodiesel production because of limited supply. Therefore, non-edible crops like *Jatropha curcas* can be used for biodiesel production. Both Government and non-government agencies in India are promoting this biodiesel crop for more than last two decades. In our country, considerable progress has been made on survey, selection, clonal propagation of *Jatropha* germplasm, large-scale cultivation in the field, and importantly biodiesel production through transesterification. However, relatively less progress has been made on studying the toxic proteins such as curcins, RIPs as found in the *Jatropha* seeds. We can develop a novel *Jatropha* plant with high curcin content in the seeds; since this toxic protein has some medicinal value in terms of antitumor effects. Earlier, the focus of our laboratory was on the propagation of *Jatropha* plant through plant tissue culture technique. Here, one of the objectives is to carry out sequence analysis and comparison using the available sequences encoding curcin isoforms. This is required to know the sequence relatedness and for predicting the significant protein motifs. This is important for both recombinant expression and viable protein engineering. Moreover, we need to adopt a suitable and easy-to-use protocol for

the isolation of genomic DNA from *Jatropha* so that structure and function of the curcin gene(s) can be studied through PCR approach using gene-specific primers. From such approach, we can assess the allelic variations between the jatropha varieties. It is also important to know the expression patterns of curcin isoforms in different tissues of *Jatropha*. A number of *Jatropha* accessions were collected from different regions of Punjab and maintained in our university campus. In this study, seven accessions of *Jatropha* i.e. TJS 17#03, TJS 42#04, TJS 35#01, TJS 01#03, TJS06#24 ,TJS19#17, TJS46#01 were chosen for molecular studies. Keeping in view of the above points the following objectives were framed.

2.10.1 Objectives of the study

- Sequence analyses, multiple sequence alignment (MSA) and motif search using the sequences of curcin isoforms at both nucleotide and amino acid level

- Analysis of amplicon profile in the different Indian *Jatropha* cultivars using their genomic DNAs and curcin-specific oligonucleotide primers

- Studies on expression patterns of curcin isoforms in *Jatropha* tissues

CHAPTER-3 MATERIALS & METHODS

3.1 *Jatropha* plant materials

For DNA isolation, leaf samples of *Jatropha* plant were collected from the different *Jatropha* cultivars such as TJS 17# 03, TJS 04 # 42, TJS 35 # 01, TJS 01 # 03, TJS 19 # 17, TJS 46 # 01, TJS 06 # 24 maintained at *Jatropha curcas* L. germplasm bank, COS-complex, Thapar University, Patiala.

3.2 Other materials

Various enzymes used were purchased from Bangalore Genei Pvt.Ltd., Bangalore. The chemicals required were bought from Siga-aldrich India Pvt. Ltd, and Himedia Pvt. Ltd, Mumbai. Primers used were synthesized by Eurofilms Genomic Pvt Ltd, Banglore. The gel extraction Qiagen kit was purchased from Genetix.

Buffers and other chemical and Enzymes

- *Gel loading buffer (5X)*

  Sucrose - 35 % (w/v)
  EDTA - 50 mM (pH 8.0)
  Tris – 25 mM
  Bromophenol blue - 0.2 % (w/v)

- *TBE (5X) buffer*

  Tris Base - 54 g L^{-1}
  Boric acid - 28 g L^{-1}
  EDTA - 3.8 g L^{-1}
  The pH of the buffer was set at 8.0

- *TE buffer*

  Tris.HCl - 10 mM (pH 8.0)
  EDTA - 1 mM (pH 8.0)

- *Extraction buffer*

  50 mM Tris-HCL (pH8.0)
  50 mM EDTA
  250 mM Nacl
  15 % Sucrose

- *Ethanol*
- *Isopropanol*
- *5 M potassium acetate solution*
- *Sodium acetate*

- *RNase*
- *Alcohol*
- *Chloroform*

3.3 Methods

3.3.1 Isolation of genomic DNA from the *Jatropha* varieties

Procedure: Genomic DNA was isolated from the *Jatropha* leaves by the protocol described by Kumari et al. (2012). Plant leaf samples were washed in tap water followed by sterile distilled water. To remove the excess water on the leaves blotting filter paper was used. 0.7 g of plant material was weighed and fine powder was made in the presence of liquid nitrogen with the help of mortar and pestle. The fine powder was then transferred to 20 mL tube containing 5 mL of extraction buffer and 0.5 % SDS (250 µL of SDS) maintained at 65°C. Contents were mixed properly with intermittent gentle shaking and incubated at 65°C for 15 min. The solution in the tube was spun at 5500 rpm for 10 minutes. Then 1/3rd volume of 5.0 M potassium acetate solution was added, mixed vigorously and incubated further on ice for 20 min and then centrifuged at 5500 rpm at 4°C for 25 min. The supernatant was filtered through a fine muslin cloth and isopropanol in equal volume was added, mixed gently and incubated at -20°C for overnight. DNA was extracted by centrifugation at 12,000 rpm at 4°C for 25 min. The crude DNA pellet was washed with ice cold 70% ethanol, air dried and suspended in 50 µL of TE buffer and stored at -20°C.

3.3.2 Purification of *Jatropha* genomic DNA

Materials Required:

- Sterile water
- DNase-free RNase enzyme
- 3.0 M sodium acetate (pH 5.5)
- Ethanol
- TE buffer
- Extraction buffer
- 8M Licl
- DEPC-treated water
- Sodium acetate

Procedure: Further purification of DNA was done by treatment with DNase–free RNase. 350 µL of sterile water was added into eppendrof containing DNA sample to make the total volume 400 µL. Then 3µL of DNase–free RNase enzyme was added into solution. The solution

was incubated for 45 minutes at 37°C. Equal volume of phenol and chloroform (200 µL each) was added into the solution and mixed properly for 10-15 minutes by gently inversion mixing. Then the sample was centrifuged at 8000 rpm for 10 minutes. DNA was precipitated using $1/10^{th}$ (0.1) volume of 3.0 M sodium acetate (pH 5.5) and double the volume of ethanol. DNA was finally recovered by dissolving the pellet in 50 µL of TE buffer and stored at (-20°C). The quality and quantity of DNA was checked spectrophotometrically by measuring the A_{260}/A_{280} ratio.

3.3.3 Isolation of RNA from different tissues of *Jatropha*

Procedure: For total RNA isolation, various tissues like leaf, stem, seed kernel and seed pericarp were harvested from candidate plus trees (CPT's) of *Jatropha* maintained in our Thapar University. From each CPT, mature and dried fruits were harvested; then the seeds were separated manually from the fruits, clean through winnowing, and kept in muslin cloth bags under ambient conditions. Subsequently in order to analyse several seed characteristics, the *Jatropha* seed samples were dried under sun for 1-2 days till they attained constant weight.

Material Required:

Table 3 Stock solution and working solution for RNA isolation

Solution	Stock Solution	Working Solution
Tris Buffer	0.5M (pH 8.0)	100mM (pH 8.0)
LiCl	8M	100Mm
EDTA	0.5M (pH 8.0)	10mM (pH 8.0)
SDS	10%	1%
B-mercaptoethanol	0.2%	0.1ml

Procedure: Plant tissues contain high amount of nucleases, polysaccharides, phenolics and other storage material. Therefore, RNA isolation from plant materials is relatively difficult in terms of intactness and quality. For that, numbers of methods are reported in literature. Here we used, SDS-Phenol method by Gilman (1987) which was used as such or with modifications depending upon the plant material.

The plant material (0.2-1.0 g) was frozen and pulverized in liquid nitrogen with the help of mortar and pestle to a fine powder. The contents were mixed in a buffer containing lithium chloride and SDS (RNA extraction buffer:100mM LiCl, 100mM Tris-HCL pH 8.0, 10mM EDTA pH 8.0, 1% SDS, 0.2% β-mercaptoethanol) followed by direct extraction with phenol choloroform (1:1). Under ice cold conditions, $1/3^{rd}$ volume of 8M licl was added for the precipitation of RNA. The crude RNA was further purified by RNase-free DNase treatment

followed by solvent extraction and ethanol precipitation. After that RNA was dissolved in RNase-free deionized water and kept in aliquots at -70°C for further use.

3.3.4 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Materials Required:

- DEPC water
- oligo dT primer
- 10 mM DNTP
- RNase inhibitor
- Reaction buffer
- RNA sample

Procedure: First strand cDNA was synthesized using Revert Aid H Minus M-MuLV reverse transcriptase. The enzyme lacks ribonuclease H activity specific to RNA in RNA-DNA hybrids. Therefore degradation of RNA does not occur during first strand cDNA synthesis, resulting in higher yields of full-length cDNA from long templates upto 13 kb. Reverse transcription (RT) was performed using the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit from Fermentas Life Sciences containing M-MuLV reverse transcriptase and the gene-specific reverse primers, JCuPR-R1590, R2CU3021. For each RT reaction, approx. 2.0 µg of total RNA either from stem or leaf from *Jatropha* cultivars was used as template. All the steps of reverse transcription were carried out according to the manufacturer's instructions. In order to isolate full-length cDNA, PCR was carried out using the individual RT product as template. 6µl DEPC water was taken and RNA sample was added. Oligo dT primer was added and mixed properly and eppendorfs were kept at 65°C for 5 min and then at room temperature for 5 min. Reaction buffer (4µl), RNase inhibitor (1µl) were added and 10mM dNTPs was added. The tubes were incubated at 42°C for 1 hour. After initial denaturation at 94ºC for 1 min 30 s, the thermal cycling parameters during PCR were: denaturation at 94ºC for 1 min, annealing at 55ºC for 2 min; polymerization at 72ºC for 3 min for 30 cycles followed by final extension at 72ºC for 5 min.

3.3.5 Designing of Oligonucleotide primers

The following oligonucleotide primers were designed based on the available genome sequence corresponding to Curcin2A gene in GenBank database (Accession no: GQ925453) and curcin precursor in GenBank database (Accession no: AF469003).

Curcin2A gene sequence of 3748 bps is comprised of the following structural features: It consist of 1790-bp 5′ flanking region (including promoter) and the transcriptional start site is

located at the base 1791 corresponding to first exon. So, the above gene sequence provides an extended Curcin2A gene promoter. The translational start site is present at the base 2085. In curcin2A gene, the ORF of polypeptide spans from the base 2085 to 3014.

Curcin precursor gene sequence of 1802 bps is comprised of the following structural features: It consists of 694-bp 5′ flanking region (including promoter) and the transcriptional start site is located at the base 695. The translational start site is present at the base 1576. In Curcin precursor gene, the ORF of polypeptide spans from the base 695 to 1576.

3.3.6 Polymerase Chain Reaction (PCR)

Material Required:

Composition of PCR reaction: Total reaction volume: 50 µL (Table 4)

Table 4 composition of PCR reaction

Material	Volume taken	Concentration
Template DNA	1 Ml	3µL
Buffer 10X	5 µL	5µL
Forward primer	2 µL	10 pmoles
Reverse primer	2 µL	10 pmoles
DNTPs	2.8µL	2.5 Mm
Sterile deionized water	volume made up to 50 µL	volume made up to 50 µL
Taq DNA polymerase	0.7µL	3 U/µL

Procedure: PCR is used to amplify a specific DNA sequence in a simple, rapid and automated manner using forward and reverse primer. PCR is repeated cycling of three steps: heat denaturation of template DNA, annealing of oligonucleotide primers to complementary sequences in the single strand template DNA, and extension of annealed primers by a thermo stable DNA polymerase. The thermal cycling parameters are given in Table 5.

Table 5 Thermal cycling parameters

Step	Temperature	Time
Denaturation	94°C	1 min
Annealing	55 °C	2 min
Polymerization	72°C	2 min

The reaction was carried out for 30 cycles with final extension at 72°C for 5 min.

3.3.7 Agarose gel electrophoresis

Materials Required

- Agarose (Himedia)

- 0.5 X TBE buffer
- Ethidium bromide dye (0.5 μg mL^{-1})
- Sterile water
- DNA samples and amplicons
- Bromophenol blue dye
- Gel Electrophoresis instrument
- UV Transilluminator
- Gel documentation system (BIO-RAD)

Procedure: Agarose gel electrophoresis was performed using standard methods (Sambrook- a laboratory manual). 1.0 % agarose gel was made in 0.5X TBE buffer and ethidium bromide dye (0.5μg mL^{-1}) was added to it. Gel was then casted in the casting tray. The DNA samples were loaded in the wells after solidification of gel. Electrophoresis was carried out in 0.5X TBE (running buffer) at 2 – 5 Volt per cm till the tracking dye covered two-third of the gel length. Finally, the DNA bands were visualized under UV light.

3.3.8 Sequence analysis

The nucleotide sequence of the curcin gene was analysed by NCBI (National Center for Biotechnology Information) website (http://www.ncbi.nlm.nih.gov). For calculating the theoretical molecular weight, isoelectric point (pI) and amino acid composition of the predicted protein, the ProtParam tool of ExPASy (Expert Protein Analysis Systerm) proteomics server of the Swiss Institute of Bioinformatics (SIB:URL: http//expasy.org/tools/) was used. The different ProtScale tools of ExPASy were used for prediction of hydrophobic character (Kyte and Doolittle 1982), and the various secondary structures such as α-helix, β-sheet, β-turn and random coil. For multiple sequence analysis, the clustal omega (an EMBL-EBI sequence analysis tool: http://www.ebi.ac.uk/tools/) was used.

4.1 Sequence analysis, comparison and motif search in different curcin forms of *Jatropha*

4.1.1 Sequence features of Curcin2A gene from *Jatropha*

For this thesis work we followed 3748-bp Curcin2A gene from *Jatropha curcas* (GenBankID: GQ925453) which encodes 309 amino acid of "Curcin2A" protein which is also regarded as ribosome inactivating protein. This 3748-bp Curcin2A gene consisting of 1790-bp 5′flanking region (including promoter) and two exons i.e. 1791 to 1845 and 2074 to 3194 bp. The entire transcriptional unit spans from 1791 to 3194. The length of first exon of this gene is 55-bp i.e. from 1791 to 1845 bp. The length of only one intron is 228-bp i.e. from1846 to 2073 bp. The length of second exon is 1121-bp i.e. from 2074 to 3194 bps. Therefore the remaining portion i.e. from 3195 to 3748 bp refers to 3′ flanking region of this gene. Size of ORF in curcin2A gene is 930 bp (3014-2084) which is equal to 310 codons including stop codon. Therefore, the Curcin2A polypeptide consists of 309 amino acids. The length of 5′UTR is equal to 294 bp (including intron). Therefore, 3′UTR of Curcin2A gene is equal to is equal to 180 bp.

4.1.2 Sequence features of Curcin precursor from *Jatropha*

For this thesis work we followed 1802-bp Curcin precursor gene from *Jatropha curcas* (GenBankID: AF469003) which encodes 293 amino acid of "Curcin precursor" protein. This 1802-bp curcin precursor gene consists of 694-bp 5′flanking region (including promoter). The entire transcriptional unit spans from 695 to 1576. Therefore the remaining portion i.e. from 1578 to 1802 bp refers to 3′ flanking region of this gene. Size of ORF in curcin precursor gene is 882 bp (1576-694) which is equal to 294 codons including stop codon. Therefore, the Curcin precursor polypeptide consists of 293 amino acids. The length of 5′ UTR is equal to 294 bp. Therefore, 3′ UTR of Curcin2A gene is equal to is equal to 325 bp.

4.1.3 Sequence analysis of the different regions of curcin2A gene by BLASTn

5′-flanking region as a query sequence: The nucleotide sequence of Curcin2A gene was analyzed by NCBI BLAST tools. When BLASTn was done for 5′ flanking region of Curcin2A gene, it shows largest promoter part in all *Jatropha curcas* gene available in database as query cover is very less. So, it shows divergence in distal part of promoter region of 5′ flanking region of Curcin2A gene. The 1790-bp 5′ flanking region of Curcin2A gene was found to be close (88 % sequence identity) to a gene copy in *Jatropha curcas* clone BAC 121e10 putative curcin 2b gene (GenBank ID: GQ925454). However sequence divergence was prominent if compared

with some known curcin genes from *Jatropha*, since the sequence identity values were 99% (GenBank ID: EU195892), 99 %(GenBank ID: EF12740). Query coverage ranged from 74% to 22 % in the BLAST search data for these sequences. 5′flanking region of the Curcin2A gene was not found 100% identical with any other Curcin gene reported to date.

Coding region as a query sequence: When BLASTn was done for coding region of Curcin2A gene, it shows more homology with other gene sequences of Curcin. The 930-bp coding region was found to be identical (100% sequence identity with 100% query coverage) to a gene copy in *Jatropha curcas* curcin gene (GenBank ID: EU395775), 99 % sequence identity with 100 % query coverage to a gene copy in *Jatropha curcas* curcin-L precursor gene (GenBank ID: EU195892), 99 % sequence identity with 99% query coverage to a gene copy in *Jatropha curcas* RIP gene (GenBank ID: AY435214), 95 % sequence identity with 100% query coverage to a gene copy in *Jatropha curcas* curcin precursor gene (GenBank ID: JF357910)

4.1.4 Sequence analysis of the different regions of curcin precursor gene by BLASTn

5′-flanking region as a query sequence: The nucleotide sequence of Curcin Precursor gene was analyzed by NCBI BLAST tools. When BLASTn was done for 5′ flanking region of Curcin precursor gene, it shows small promoter part in all *Jatropha curcas* gene available in database as query cover is very less. So, it shows divergence in distal part of promoter region of 5′ flanking region of Curcin precursor gene. The 695-bp 5′ flanking region of Curcin precursor gene was found to be close (99 % sequence identity) to a gene copy in *Jatropha curcas* clone BAC 121e10 putative curcin 2b gene and curcin 1 gene (GenBank ID: GQ925454). However sequence divergence was prominent if compared with some known curcin genes from *Jatropha*, since the sequence identity values were 98% (GenBank ID: EF612739), 82 %(GenBank ID: EU195892). Query coverage ranged from 88% to 21% in the BLAST search data for these sequences. 5′flanking region of the Curcin2A gene was not found 100% identical with any other Curcin gene reported to date.

Coding region as a query sequence: When BLASTn was done for coding region of Curcin precursor gene, it shows more homology with other gene sequences of Curcin. The 882-bp coding region was found to be identical (98% sequence identity with 100% query coverage) to a gene copy in *Jatropha curcas* curcin precursor gene (GenBank ID: JF357910), 97 % sequence identity with 100 % query coverage to a gene copy in *Jatropha curcas* clone BAC 121e10 putative curcin 2b and curcin 1 gene (GenBank ID: GQ925454), 94 % sequence identity with 99% query coverage to a gene copy in *Jatropha curcas* curcin mRNA gene (GenBank

ID:FJ357424), 94 % sequence identity with 100% query coverage to *Jatropha curcas* curcin2A gene (GenBank ID: GQ925453)

4.1.5 Some biochemical attributes of Curcin2A protein (GenBank Protein ID: ADN39428)

The entire 309 amino acids sequence of curcin2A protein was analysed by using protparam tool of ExPASy resource portal under Swiss Institute of Bioinformatics (SIB) which revealed some of the important biochemical attributes. The calculated molecular weight of the curcin2A protein was found to be 34.8 kDa with a predicted isoelectric point (pI) of 6.20. Predicted formula of Curcin2A protein is $C_{1584}H_{2452}N_{404}O_{465}S_8$. Out of its total 309 amino acids, 32 were strongly basic (+) (Arg + Lys), 33 were strongly acidic (-) (Asp + Glu).The instability index (II) was computed as 29.55, which classified the protein as stable. The amino acid composition data revealed that some of the amino acids such as Asn (7.1 %), Ile (7.1%), Lys (7.4%), Phe (5.5%), Ser (8.1%), Tyr (4.5%) and Val (8.4%) occurred more frequently as compared to their average occurance; whereas the amino acids namely Arg (2.9%), cys (0.6%), Glu (5.2%), Gly (4.5%), Met (1.9%), Pro (2.9%) and Thr (4.9%) occurred less frequently (Doolittle 1989). The estimated half life of curcin2A protein is 30 h as predicted in this analysis.

Hydropathy plot of Curcin2A protein

The hydropathy profile was generated for Curcin2A protein using the ProtScale tool based on the Kyte-Doolittle scale in the given Fig. 2

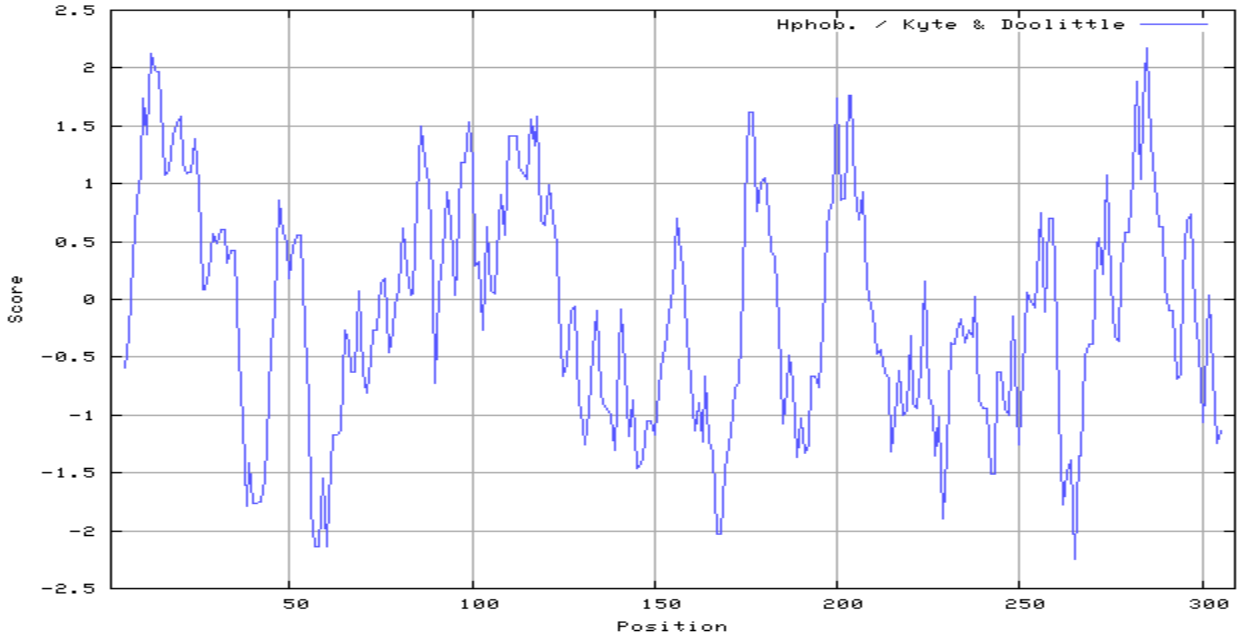

Fig. 2 Hydropathy plot of Curcin2A protein

Hydropathy plot of curcin2A protein revealed that N terminal of amino acid sequences of Curcin2A protein is Hydrophobic i.e. 5 to 30 position of amino acid sequence. Hydrophilic

regions in the plots are at 30-40, 55-65, 120-150, 160-175, 220-255 and 265-275 positions of amino acid in curcin2A protein. Hydrophobic region in the plots are at 70-115, 165-170, 190-220 and 275-300 positions of amino acid in curcin2A protein.

4.1.6 Some biochemical attributes of curcin precursor (GenBank protein ID: AAL86778)

The 293-aa Curcin precursor sequence (GenBank ID:AF469003) was analyzed by using ProtParam tool of ExPASy resource portal under Swiss Institute of Bioinformatics (SIB) which revealed couple of attributes about the deduced polypeptide. The molecular weight of predicted 293-aa polypeptide was ~32766 kDa. The total number of negatively charged residues (Asp+Glu) was 28 and the total number of positively charged residues (Arg+Lys) was 31. This data clearly indicated that the protein is basic in nature which was further proven by its theoretical pI i.e., 8.55. The amino acid composition data showed that some of the amino acids such as Ala (8.9%), Lys (7.2%) and Leu (8.2%) occurred more frequently as compared to their average occurrence; whereas amino acids Gln (0.6%), Trp (0.6%) and Tyr (1.6%) occurred less frequently (Doolittle 1989). The instability index (II) was computed to be 23.31 thus the protein was classified as stable. The average occurrence of Gly was found to be more frequent which implied that the protein showed less propensity towards the alpha-helix formation. The half life was estimated to be 30 hours

Hydropathy plot of Curcin precursor protein

The hydropathy profile was generated for Curcin precursor using the ProtScale tool based on the Kyte-Doolittle scale in the given Fig. 3

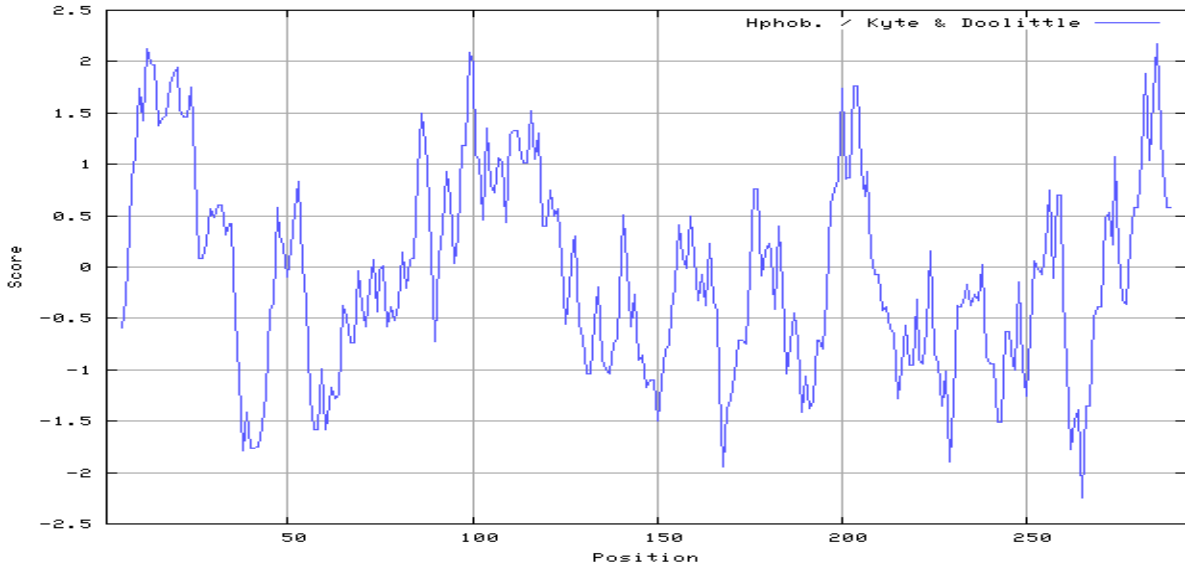

Fig. 3 Hydropathy plot of Curcin precursor protein

Hydropathy plot of curcin precursor protein revealed that N terminal of amino acid sequences of Curcin precursor is Hydrophobic i.e. 7 to 35 position of amino acid sequence. Hydrophilic regions in the plots are at 36-46, 55-72, 129-139, 143-155, 161-175, 184-196, 209-223 positions of amino acid in curcin precursor protein. Hydrophobic region in the plots are at 47-54, 91-124, 197- 207 and 275-300 positions of amino acid in curcin precursor protein.

4.1.7 BLASTp analysis of Curcin2A protein

When BLASTp was done for Curcin2A protein (GenBank protein ID: ADN39428); it shows significant homology with other forms of curcins in *Jatropha* as given in Table 6. The 309-amino acid sequence was found to be identical (100% sequence identity with 100% query coverage) to amino acid sequence in *Jatropha curcas* curcin (GenBank protein ID: ABZ04128), 99 % sequence identity with 100 % query coverage to the amino acid sequence in *Jatropha curcas* curcin-L precursor gene (GenBank protein ID: ABW17545), 99 % sequence identity with 100% query coverage to the amino acid sequence in *Jatropha curcas* RIP gene (GenBank protein ID: AAR08395), 98 % sequence identity with 94% query coverage to the amino acid sequence in *Jatropha curcas* curcin (GenBank protein ID: ACO53803). The 309-amino acid sequence of Curcin2A gene showed C terminal variation with the amino acid sequence in *Jatropha curcas* curcin (GenBank protein ID: ACO53803) as 16 amino acids are unique at C-terminal of Curcin2A.

Table 6 Details of some homologous curcin sequences available in the database

Protein name	Accession No.	ORF length	Amino acid
Curcin	ABZ04128	930	309
Curcin-L precursor	ABW17545	930	309
RIP	AAR08395	930	309
Curcin	ACO53803	882	293
Curcin precursor	AEA72440	930	309

4.1.8 BLASTp analysis of Curcin precursor

When BLASTp was done for Curcin precursor (GenBank protein ID: AAL86778); it shows significant homology with other forms of Curcin in *Jatropha* as given in Table 7. The 293-amino acid sequence was found to be identical (95% sequence identity with 100% query coverage) to amino acid sequence in *Jatropha curcas* curcin precursor (GenBank protein ID: AEA72440), 94% sequence identity with 100 % query coverage to the amino acid sequence in *Jatropha curcas putative* curcin 2b gene (GenBank protein ID: AJW31110), 89% sequence identity with 100% query coverage to the amino acid sequence in *Jatropha curcas* RIP gene

(GenBank protein ID:AAR08395), 89 % sequence identity with 100% query coverage to the amino acid sequence in *Jatropha curcas* curcin-L precursor (GenBank protein ID: ABW17545).

Table 7 Details of some homologous curcin sequences available in the database

Protein name	Accession No.	ORF length	Amino acid
Curcin Precursor	AAL86778	882	293
Curcin precursor	AEA72440	930	309
Curcin 2b	AJW31110	882	293
RIP	AAR08395	930	309
Curcin-L precursor	ABW17545	930	309

4.1.9 Significant protein motifs in Curcin2A (GenBank protein ID: ADN39428)

The predicted amino acid sequence of curcin2A was carefully examined for detecting the presence of protein motifs using following program: http://myhits.isbsib.ch/cgibin/motif_scan. A number of motifs were predicted as shown in Fig. 4, and the details are provided in Table 8.

```
MKGGKMNLSIMVAAWFCWSSIIFGWASAREIVCPFSSNQNYKAGSTPTLAITYDATTDKKNYAQFIEDLR  70
EAFDFSYLSHKIPVLRATVAANQKFIVAKVINSGDIEVSVGLNVINAYLVAYKVGSNSYFFNDSESLADA 140
KKNLFTDTNQQTLAFTGSYADFESRAKLHREEVDLGVVALDNYVYTLEKSSQPADIAKPLVGFIEMVPEA 210
ARFKYIEKKISTQISKTFRPRGDIISLENNWGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKG 280
LMGVLLNAVNYKVSMEEIIFNDQKWLPWL                                           309
```

Fig. 4 Prediction of protein motifs in curcin2A protein sequence

Four motifs were present on N- glycosylation site which are highlighted with light green colour. One cAMP and cGMP dependent protein kinase phosphorylation site was found which is highlighted with yellow colour. From Casein kinase II phosphorylation site five motifs were found which are highlighted with light grey colour. Three motifs on N-myristoylation sites were found which are blue coloured. From Protein kinase C phosphorylation site four motifs are present which are highlighted with pink colour and only one cell attachment sequence motif was found which is red in colour.

Table 8 Rrepresenting motif sites and the position of the motifs of curcin2A protein (Ref: http://myhits.isbsib.ch/cgibin/ motif_scan)

Sr No.	Motif site	Motif	Motif position
1	N-glycosylation site	NLSI	7-10
2	N-glycosylation site	NDSE	132-135
3	N-glycosylation site	NYTN	266-269
4	N-glycosylation site	NVTQ	274-277
5	cAMPand cGMP dependent protein kinase phosphorylation site.	KKIS	218-221
6	Casein kinase II phosphorylation site.	SARE	27-30
7	Casein kinase II phosphorylation site	SLAD	136-139
8	Casein kinase IIphosphorylation site	SYAD	158-161
9	Casein kinase II phosphorylation site	SVDD	251-254
10	Casein kinase II phosphorylation site	SMEE	294-297
11	N-myristoylation site	GGKMNL	3-8
12	N-myristoylation site	GSTPTL	44-49
13	N-myristoylation site	GVLLNA	283-288
14	Protein kinase C phosphorylation site	SAR	27-29
15	Protein kinase C phosphorylation site	TDK	57-59
16	Protein kinase C phosphorylation site	SHK	79-81
17	Protein kinase C phosphorylation site	TFR	227-229
18	Cell attachment sequence	RGD	231-233

4.1.10 Significant protein motifs in curcin precursor (GenBank protein ID: AAL86778)

The predicted amino acid sequence of curcin precursor was carefully examined for detecting the presence of protein motifs using following program: http://myhits.isbsib.ch/cgibin/motif_scan. A number of motifs were predicted as shown in Fig. 5 and the details along are provided in Table 9

```
MKGGKMNLSIMVAAWFCWSCIIFGWASAREIVCPFSSNQNYKAGSTPTLTITYDAAADKKNYANFIRDLR 070
EAFGFSYSSHEIPVLRATVAANQKFIVAKVINVANLEVSLGLNVVNAYLVGYKVGGTSYFFNDPESLADA 140
KTYLFTDTKQQTLSFTGSYADFLSRANVHREDVDLGVQALDNYIYTLEKSSKPADIAKPLVGFIEMVPEA 210
ARFEYIEKKISTQISKTFRPRGDIISLENNWGDLSYQIQKSVDDVFLKPVQLQRENYTNILVNNVTQVKG 280
LMGVLLNAVKYKV                                                          293
```

Fig. 5 Prediction of protein motifs in curcin precursor

Three motifs were present on N- glycosylation site which are highlighted with light green colour. One cAMPand cGMP dependent protein kinase phosphorylation site was found highlighted with yellow colour. From Casein kinase II phosphorylation site five motifs were found which are highlighted by light grey colour. Three motifs on N-myristoylation sites were found which are blue coloured. From Protein kinase C phosphorylation site three motifs were present which are highlighted with pink colour and only one cell attachment sequence motif was found which is red in colour.

Table 9 representing motif sites and the position of the motifs of curcin precursor (Ref: http://myhits.isbsib.ch/cgibin/ motif_scan)

Sr No.	Motif site	Motif	Motif position
1	N-glycosylation site	NLSI	7-10
2	N-glycosylation site	NYTN	266-269
3	N-glycosylation site	NVTQ	274-277
4	cAMPand cGMP dependent protein kinase phosphorylation site.	KKIS	218-221
5	Casein kinase II phosphorylation site.	SARE	27-30
6	Casein kinase II phosphorylation site	SSHE	78-81
7	Casein kinase II phosphorylation site	SLAD	136-139
8	Casein kinase IIphosphorylation site	SYAD	158-161
9	Casein kinase II phosphorylation site	SVDD	251-254
10	N-myristoylation site	GGKMNL	3-8
11	N-myristoylation site	GSTPTL	44-49
12	N-myristoylation site	GVLLNA	283-288
13	Protein kinase C phosphorylation site	SAR	27-29
14	Protein kinase C phosphorylation site	SSK	190-192
15	Protein kinase C phosphorylation site	TFR	227-229
16	Cell attachment sequence	RGD	231-233

4.1.11 Multiple sequence alignment (MSA) of different curcins from *Jatropha*

A total of five curcin protein sequence from *Jatropha*, i.e., Curcin precursor (Cu AEA72440), ribosome-inactivating protein cucurmosin-like, *Jatropha* curcas (RIP cu-like XP_012074358) Ribosome-inactivating protein partial, *Jatropha curcas* (RIP , AAR08395), Curcin2A (cu2A, ADN39428) and Curcin-L precursor (Cu-L, ABW17545), were included in the multiple sequence alignment (Fig.6). Minor manual adjustments were also made during alignment. This study was done to examine sequence similarities and divergence, the amino acid substitutions, insertions and deletions between the five different forms of curcin proteins.

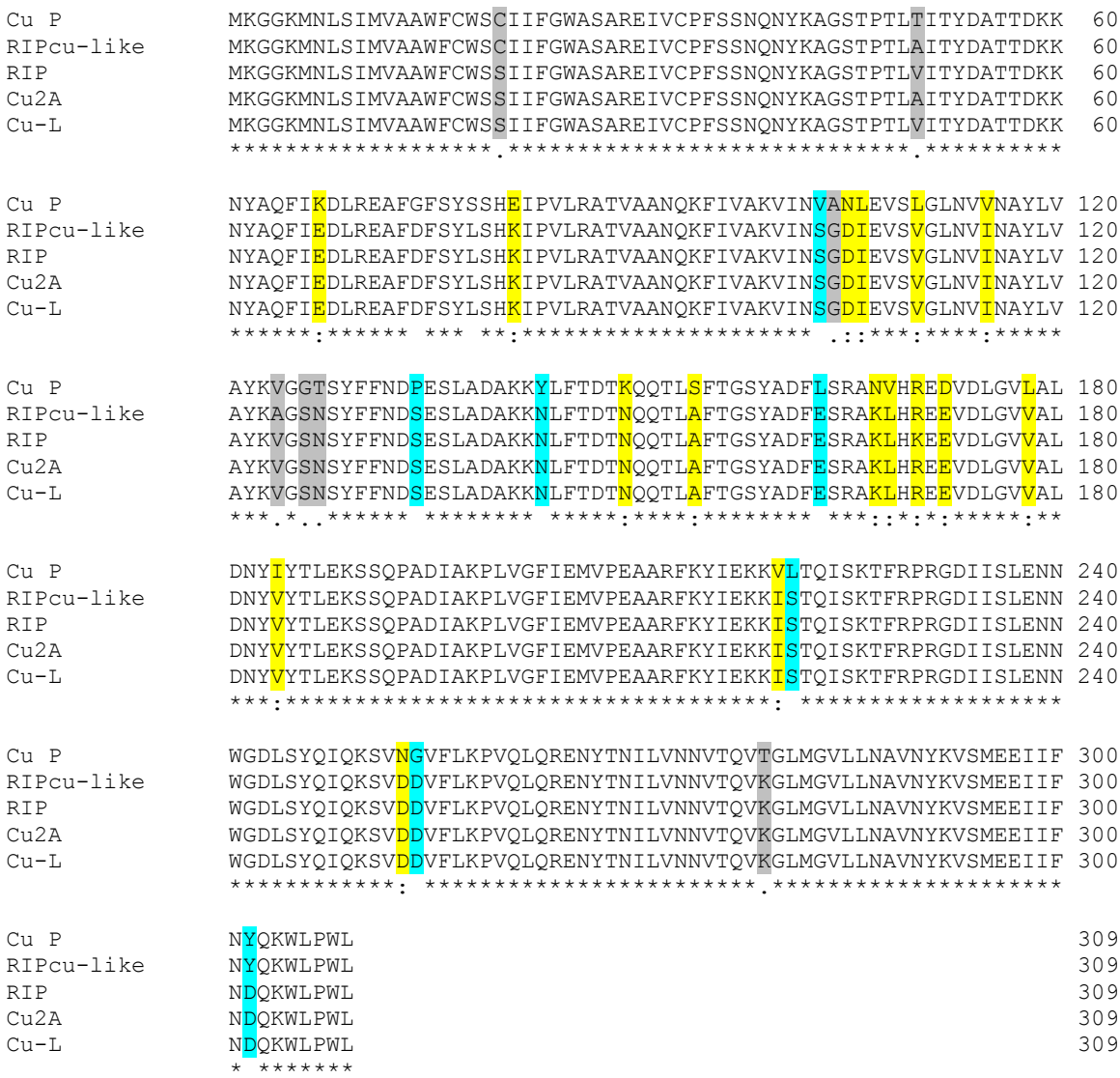

Fig. 6 Multiple sequence alignment of the deduced amino acid sequences of five different forms of Curcin protein from the *Jatropha* cultivars (see the text for GenBank protein ID).This alignment is based on Clustal omega tool. The star symbols denote the conserved amino acid between all the four forms of curcin proteins studied. Alignment of Curcin protein showed some divergence with respect to the other 4 forms of curcin protein which is highlighted with different colours.

In the N-terminal of the all the curcin forms the amino acids were nearly conserved with minor variations. Considering Curcin2A protein as a reference, Curcin P was found to vary at 30 locations, mostly conserved mutations found are E67K, K81E, D106N, I107L, V111L, I116V, N148K, A154S, K167N, L168V, R170K, E172D, V178L, V184I, I220V and D254N which are highlighted with yellow colour. The semi-conserved mutations were found at position S20C, V50T, G114A, V124A, S126G, N127T and K229T which are highlighted with light grey colour and the non-conserved mutations were highlighted with light blue and were found at position S113V, S134P, N143Y, E161L, S221L, D255G and D302Y.

4.2 Analysis of amplicon profile in various *Jatropha* cultivars using genomic DNA

4.2.1 Isolation of genomic DNA from *Jatropha* accessions

Total genomic DNA was isolated from the leaf samples collected from the field grown *Jatropha* plants of the following accessions: TJS 17#03, TJS 42 #04, TJS 35#01, TJS 19#17, TJS 06#24, TJS 01#13 and TJS 01#03 (see section 3.3.1 of 'Materials & Methods'). The quality of genomic DNA was checked by agarose gel electrophoresis as shown in Fig. 7. The quality and quantity of DNA was also checked spectrophotometrically as presented in Table 10.

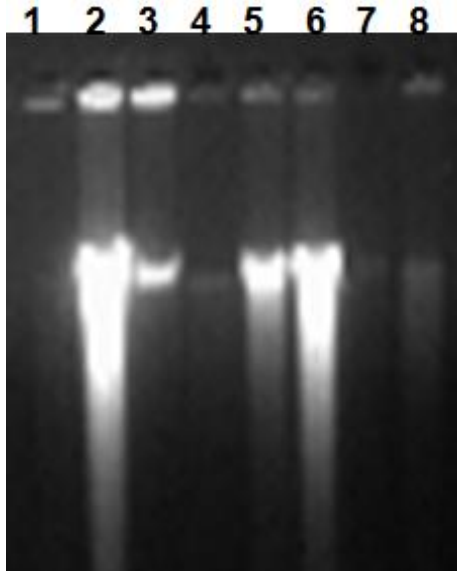

Fig. 7 Genomic DNAs from *Jatropha* varieties. Lane wise data Lane 1- TJS01#13; Lane 2-TJS 17#03, Lane 3- TJS 42#04 , Lane 4- TJS 46#04 ,Lane 5- TJS 35#01, Lane 6- TJS 19#17, Lane 7- TJS 06#24, Lane 8- TJS 35#01

Table 10 Quantification of *Jatropha* DNA using nanodrop spectrophotometer

***Jatropha* Cultivar**	**Amount of Material (g)**	**Volume of DNA soln. (µL)**	**Absorb. Ratio (A_{260}/A_{280})**	**Conc. (ng/µL)**
TJS17#03	0.7	50	1.23	920
TJS04#42	0.7	50	1.09	420
TJS35#01	0.7	50	1.00	810
TJS01#03	0.7	50	1.04	290
TJS19#17	0.7	50	1.00	810
TJS06#24	0.7	50	1.04	620
TJS46#01	0.7	50	1.35	600

4.2.2 Designing of the oligonucleotide primers

To ensure minimum chances of non-specific amplification, important factors kept in consideration while designing the primers were:

- Oligonucleotide primers should be 10-24 nucleotides long.
- GC content should be 40-60%.
- The primer should not be self-complementary or complementary to any other primer to form primer-dimer or hair pin.
- Melting temperatures of primer pairs should not differ by more than 5ºC, so the GC content and length must be chosen accordingly.
- The annealing temperature should be about 5°C lower than the melting temperature.
- Long run sequences of a single nucleotide should be avoided.
- Primers with significant structures are avoided

The features of forward primers (each 20 mer) specific for Curcin2A genes and curcin precursor are briefly discussed below:

One forward primer and one reverse primer were designed from coding regions of curcin2A gene from the accession no: GQ925453

- F4-CU2075 is a forward primer that corresponds to bases 2075-2094 of the genome sequence i.e., 5′-TGAAATCAATATGAAAGGTG-3′
- R2-CU3021 is a reverse primer that is complementary to the bases 3002-3021 of the genome sequence i.e., 5′-AGTAGGATTAAAGCCATGGC-3′

One forward primer and one reverse primer were designed from coding regions of curcin precursor gene from the accession no: AF469003

- JCuPR-F672 is a forward primer that corresponds to bases 672-691 of the genome sequence i.e. 5′-ACAACTGGACAGGTGAAATC-3′
- JCuPR-R1590 is a reverse primer that is complementary to the bases 1571-1590 of the genome sequence i.e., 5′-AATAATTTCTTCCATTCAGAC-3′

Details of Curcin 2A and curcin precursor gene specific primers:

F4-CU2075: This primer sequence encompasses the coding region only and which appears to be conserved in a no. of curcin and curcin-related genes along with Cucurmosin like protein (Ribosome inactivating protein) as reported in the database.

R2-CU3021: This sequence also appears to be conserved with many other curcin gene sequences as reported from *Jatropha curcas*.

JCuPR-F672: This primer sequence encompasses the coding region only and which appears to be conserved in a no. of curcin and curcin-related genes as reported in the database

JCuPR-R1590: This sequence also appears to be conserved with many other curcin gene sequences as reported from *Jatropha curcas*.

4.2.3 PCR using Curcin2A and Curcin precursor gene-specific primer pairs

One of the major objectives of the study is to amplify curcin gene(s) preferably with the coding regions from a few *Jatropha* accessions through PCR approach using specific primers. For this purpose, genomic DNA preparations from the following *Jatropha* accessions namely TJS17#03, TJS42#04, TJS19#17, TJS06#24, TJS46#01 TJS35#01 and TJS01#03 were used during PCR. For each specific primer pair, PCR was carried out under annealing temperature 55°C. Individual primer pair-specific amplicon profiles are shown in the following sections.

Results of curcin2A-specific PCR: PCR amplification data using different *Jatropha* genomic DNAs and the primer pair (F4-CU2075, R2-CU3021) is shown in Fig. 8. Amplifications occurred in all cases of *Jatropha* cultivars except two namely TJS19#17 and TJS01#13 where the size of amplicon was around ~1.0 kb in each case as expected (Table 11). The result suggests that curcin2A gene is conserved in these cultivars. However, there might be minor sequence divergence which could only be revealed through sequencing. The above primers did not work for some cultivars possibly due to considerable sequence divergence/allelic variations which needs to be understood.

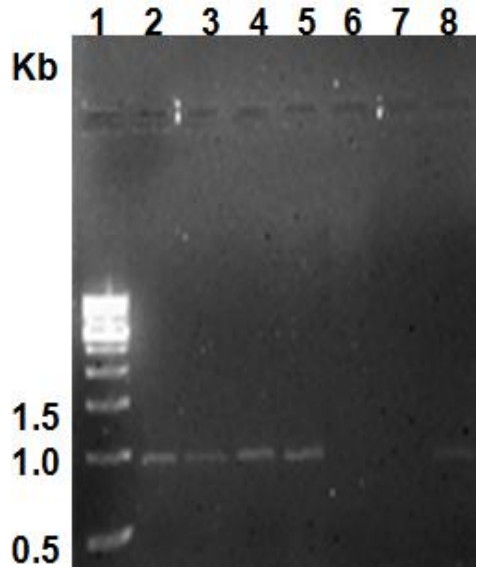

Fig. 8 PCR amplified products using gene specific primer pairs (F4-CU2075 & R2-CU3021). Annealing temp 55°C *Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 17#03; *Lane* 3- PCR amplified band of TJS 42#04; *Lane* 4- PCR amplified band of TJS 06#24; *Lane* 5- PCR amplified band of TJS01#04; *Lane* 6- PCR amplified band of TJS 35#01. *Lane* 6- PCR amplified band of TJS19#17; *Lane* 7-- PCR amplified band of TJS01#13; *Lane* 8-- PCR amplified band of TJS 17#03

Table 11 F4-CU2075 & R2-CU3021 primer pair-specific amplicons

Jatropha **Accessions**	**Size of amplicons (annealing temp. 55°C)**
TJS 17#03	~1.0 kb
TJS 42#04	~1.0 kb
TJS 06#24	~1.0 kb
TJS01#04	~1.0 kb
TJS 35#01	~1.0 kb
TJS19#17	No bands
TJS01#13	No bands
TJS17#03	~1.0 kb

Results of curcin precursor-specific PCR: PCR amplification data using different Jatropha genomic DNAs and the primer pairs (JCUPR-F672 & JCUPR-R1590) is shown in Fig. 9. Amplifications occurred in all cases of *Jatropha* cultivars except one namely TJS19#17 where the size of amplicon was around ~0.9 kb in each case (Table 12). In the cases of TJS 17#03 and TJS01#04 an additional amplicon of ~0.8 kb was found. The results suggest that curcin precursor gene is mostly conserved in these cultivars. However, it is likely that the genotypic variations exsist in these cultivars. This needs to be substantiated by further study.

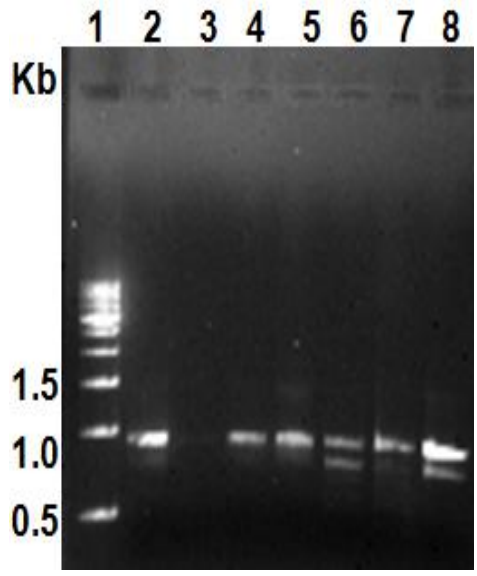

Fig. 9 PCR amplified products using gene specific primer pairs (JCUPR-F672, JCUPR-R1590)**.** Annealing temp 55°C *Lane* 1- 500 bp ladder*; Lane* 2- PCR amplified bands of TJS 35#01; *Lane* 3- PCR amplified band of TJS 19#17; *Lane* 4- PCR amplified band of TJS 01#13; *Lane* 5- PCR amplified band of TJS 06#24; *Lane* 6- PCR amplified band of TJS 17#03.; *Lane* 6- PCR amplified band of TJS 42#04; *Lane* 7-- PCR amplified band of TJS01#04; *Lane* 8-- PCR amplified band of TJS 01#46

Table 12 JCUPR-F672 & JCUPR-R1590 primer pair-specific amplicon

Jatropha **Accessions**	**Size of amplicons (annealing temp. 55°C)**
TJS 35#01	~0.9 kb
TJS 19#17	~0.9 kb
TJS 01#13	~0.9 kb
TJS 06#24	~0.9 kb
TJS 17#03	~0.9 kb, ~0.8 kb
TJS 42#04	~0.9 kb
TJS01#04	~0.9 kb, ~0.8 kb

4.3 Preliminary expression studies in different *Jatropha* tissues

4.3.1 Isolation of RNA from *Jatropha* accessions

Total RNA was isolated from the various *Jatropha* tissues i.e. leaf, seed kernel, seed pericarp and stem samples collected from the field grown plant (see section 3.3.3 of 'Materials & Methods'). The quality of RNA was checked by agarose gel electrophoresis as shown in Fig. 10. Distinct ribosomal RNA bands are there indicating intactness of the total RNA preparations.

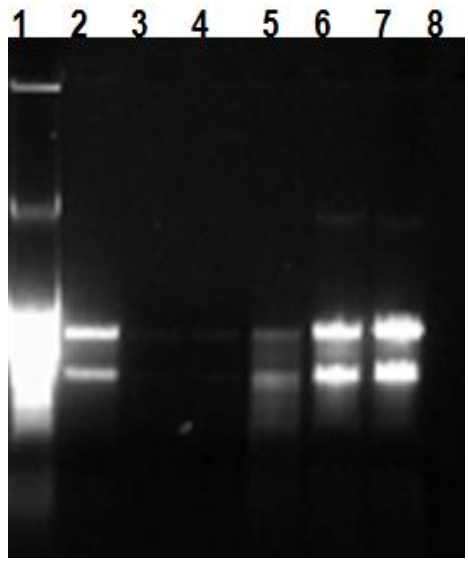

Fig. 10 RNA isolation from various *Jatropha* tissues. Lane 1- Leaf ; Lane 2-seed kernel 1, Lane 3- seed kernel 2, Lane 4- seed kernel 3 ,Lane 5-stem, Lane 6-seed pericarp 1, Lane 7-seed pericarp 2, Lane 8- seed pericarp 3

The quality and quantity of RNA was also checked spectrophotometrically by measuring the A_{260}/A_{280} ratio as presented in Table 13

Table 13 Quantification of *Jatropha* RNA using nanodrop spectrophotometer

***Jatropha* Tissue**	**Amount of Material (g)**	**Volume of RNA soln. (µL)**	**Conc. (µg/µL)**
Stem	0.7	50	210 µg
Leaf 1	0.7	50	1010 µg
Leaf 2	0.7	50	840 µg
Leaf 3	0.7	50	660 µg
Seed kernel 1	0.7	50	220 µg
Seed kernel 2	0.7	50	350 µg
Seed kernel 3	0.7	50	530 µg
Seed Pericarp 1	0.7	50	120 µg
Seed pericap 2	0.7	50	480 µg
Seed pericarp 3	0.7	50	730 µg

.

4.3.2 Purification of RNA

Purified RNA samples from different *Jatropha* tissues were checked by agrose gel electrophoresis as shown in Fig. 11. Distinct ribosomal RNA bands were seen in the gel indicating the intactness of total RNA preparation.

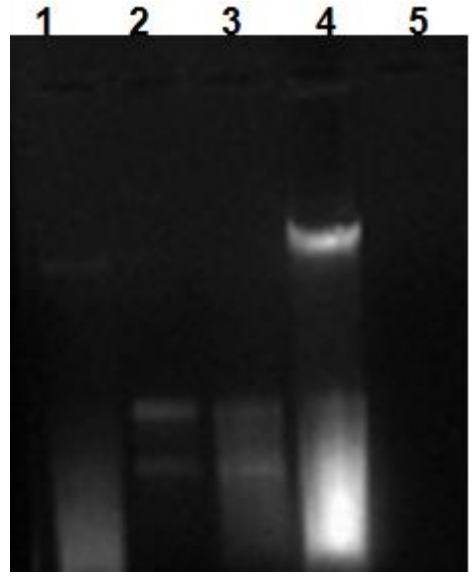

Fig. 11 Purified RNA from various *Jatropha* tissues. Lane wise data Lane1- stem lane 2-seed kernel lane 3- seed pericarp lane 4- leaf

4.3.3 RT PCR using curcin2A and curcin precursor for different tissues:

One of the major objectives of the study is to check the preliminary studies of different tissues of *Jatropha* plant using RT PCR approach using specific primer pairs. For this purpose, the *Jatropha* accession TJS01#04 was used for the isolation and purification of RNA for different

tissues. RT-PCR was carried out using oligo dT primer. Individual profiles are shown in the following sections.

Results of RT-PCR: RT-PCR was carried out using total RNA from different *Jatropha* tissues and the primer (R2-CU3021). The RT-PCR data clearly indicated that amplifications occurred only in the cases of three *Jatropha* tissues namely seed pericarp and seed kernel where the size of amplicon was around ~1.0 kb (Fig. 12) which was in the expected size range. However, the seed pericarp shows more prominent band as compared to the other tissues. RT-PCR data suggest that the level of curcin2A expression is relatively higher in seed pericarp as compared to seed kernel and stem.

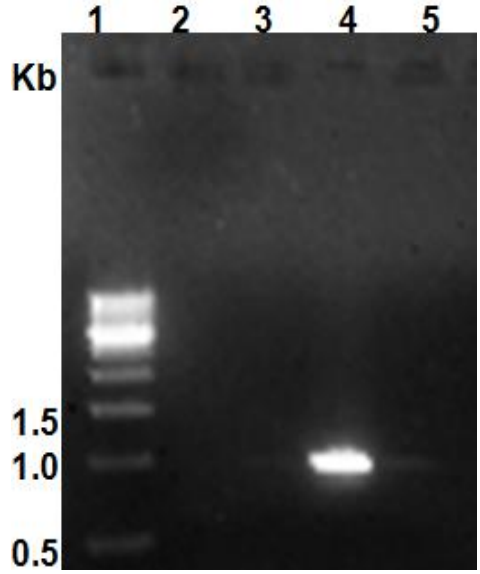

Fig. 12 RT PCR products using OLIGO dT primer (R2-CU3021). Lane wise data *Lane* 1- 500 bp ladder*; Lane* 2-Stem tissue; *Lane* 3-Seed kernel; *Lane* 4-Seed pericarp; *Lane* 5-Seed pericarp 2

Table14 R2-CU3021 primer RT PCR

***Jatropha* tissues**	**Size of RNA**
Stem	No band
Seed kernel	~1.0 kb
Seed pericarp 1	~1.0 kb
Seed pericarp 2	~1.0 kb

Results of RT-PCR: RT-PCR was carried out using total RNA from different *Jatropha* tissues and the primer (JCuPR-R1590). The RT-PCR data clearly indicated that amplifications occurred only in the case of *Jatropha* tissues namely seed pericarp where the size of amplicon was around ~1.0 kb (Fig. 13) which was in the expected size range (Table 15). It is likely that curcin precursor is differentially expressed in *Jatropha* tissues.

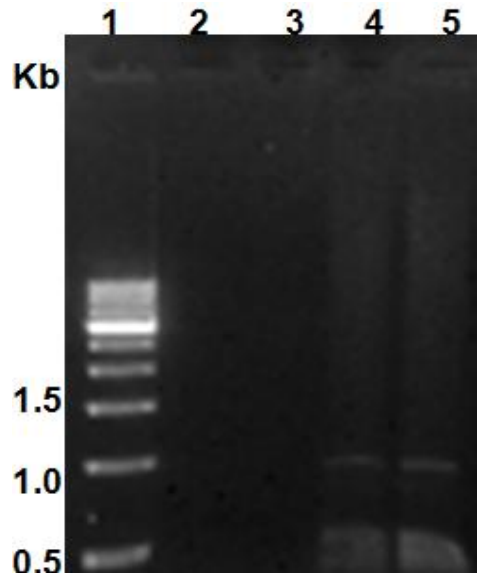

Fig. 13 RT PCR products using OLIGO dT primer (JCuPR-R1590). Lane wise data *Lane* 1- 500 bp ladder*; Lane* 2-Stem tissue; *Lane* 3-Seed kernel; *Lane* 4-Seed pericarp 1; *Lane* 5-Seed pericarp2

Table 15 JCuPR-R1590 primer RT PCR

***Jatropha* tissues**	**Size of amplicon RNA**
Stem	No band
Seed kernel	No band
Seed pericarp 1	~1.0 kb
Seed pericarp 2	~1.0 kb

- There are some curcin sequences available in the database at both nucleotide and amino acid level. In this study, sequence analysis and comparison were made using curcin2A and curcin precursor sequences at both nucleotide and amino acid level. This exercise provided a clear and comprehensive idea about the sequence relatedness between the various curcin isoforms.
- Multiple sequence alignment of the predicted polypeptides of different forms of curcin such as curcin, curcin-L precursor, curcin2A, RIP cucurmosin-like and RIP clearly revealed that some forms are highly conserved (nearly identical) whereas in some other forms of curcin considerable sequence variation could be found in terms of both conservative and non-conservative mutations of amino acids. Therefore it is likely that various curcin forms could differ with regard to their overall functionalities.
- A number of forward and reverse gene-specific oligonucleotide primers were made based on curcin2A and curcin precursor gene sequences.
- PCR was carried out using different combinations of primers. A number of amplicons as obtained in the study appeared to be quite promising. Further molecular cloning and sequencing of some of the amplicons could identify some new allelic variants of curcin gene from our *Jatropha* accessions.
- Total RNA was isolated and purified from different tissues of *Jatropha* grown under field conditions.
- Efforts were made to study expression patterns of curcin2A and curcin precursor in different tissues of *Jatropha* using RT-PCR approach. The expression in curcin2A was observed more prominent in seed pericarp than seed kernel; in the case of curcin precursor, the expression was noticed in seed pericarp only. This data suggest differential expression patterns of curcin isoforms in *Jatropha.*
- This thesis work would be useful for further studying curcin isoforms particularly in the *Jatropha* accessions suitable to the Indian agro-climatic conditions.

REFRENCES

- Achtena WMJ, Verchotb L, Frankenc YJ, Mathijsd E, Singhe VP, Aertsa P, Muysa B (2008) *Jatropha* bio-diesel production and use. Toxicology: 1063 – 108
- Adams EI and Magzoub M (1975) Toxicity of *Jatropa curcas* on goats. Toxicology: 4347-4354
- Aderibigbe AO, Johnson COLE, Makkar HPS, Becker K (1997) Chemical composition and effect of heat on organic matter and nitrogen degradability and some anti-nutritional components of *Jatropha* meal. Animal Feed Sci Technol 67:223-243
- Ahmed WA, Salimon J (2009) Phorbol Ester as Toxic Constituents of Tropical *Jatropha Curcas* Seed Oil 31(3):429-436
- Aregheore EM, Makkar HPS, Becker K (1998) Assessment of lectin activity in a toxic and a non-toxic variety of *Jatropha curcas* using latex agglutination and haemagglutination methods and inactivation of lectin by heat treatments. J Sci Food Agric 77:349-352
- Baranwal BK, Sharma MP (2005) Prospects of biodiesel production from vegetable oils. Renew. Sustain. Energy reviews 9:363-378
- Barbieri L, Battelli M, Stirpe F (1993) Ribosome-inactivating protein from plants. Biochim Biophy Acta 1154:237-282
- Battelli MG (2004) Cytotoxicity and toxicity to animals and humans of ribosome-inactivating proteins. Mini Rev Med Chem 4:513–521
- Dehgan B (1984) Phylogenetic significance of interspecific hybrid-ization in *Jatropha* (Euphorbiaceae).Syst Bot 9:467–478
- Devappa RK (2010) Biodegradation of *Jatropa curcas* phorbol esters in soil. J Sci food agric 90(12):2090
- Doolittle RF (1989) Redundancies in protein sequences. In: FasmanGD (ed) Prediction of protein structure and the principles of protein conformation. Plenum Press, New York, pp 599–623
- Duke JA (1988) CRC Handbook of Medicinal Herbs. CRC Press, Boca Raton, FL, pp. 253–254
- Fairless D (2007) The little shrub that could-may be. Nature 449:652-655
- Felke J (1914) The poisonous principles of the seeds of *Jatropha curcas* Linn. Landw
- Frankel AE, Houston LL, Fathman G (1986) Prospects for immunotoxin therapy in cancer. Annu Rev Med 37:25- 42
- Goel G, Makkar HPS, Francis G. and Becker K. (2007) Phorbol esters: Structure, biological activity and toxicity in animals. *Int J Toxicol* 26:279–288.
- Gofferje G, Schmid M and Stabler A (2015) Characterization of *Jatropha curcas* L. Protein Cast Films with respect to Packaging Relevant Properties Anthelmintic Vol. 2(3), pp. 47-49

- Gubitz GM, Mittelbech M, Trabi M (1999) Exploitation of tropical oil seed plant *Jatropha curcas* L. Bioresour. Technol. 67, 73–82.
- Haas W, Sterk H, and Mittelbach M (2002) 12-deoxy-16-hydroxyphorbol diesters isolated from the seed oil of *Jatropa curcas*. J Nat Prod 65:1434–1440
- Heller J (1996) Promoting the conservation and use of underutilized and neglected crops.1.Physic nut *Jatropha curcas* L. Int Plant Genetic Resources Insti Rome pp. 66
- Jones N, Miller JH (1991) *Jatropha curcas*: A multipurpose species for problematic sites. Land Resour Ser 1:1-12
- Juan L, Fang Y, Lin T, Fang C (2003) Antitumor effects of curcin from seeds of *Jatropha curcas.* Acta Pharmacol Sin 24 (3): 241 -246
- Jummai AT, Okoli BJ (2014) Curcin from *Jatropha curcas* seed as a potential Anthelmintic.Advance. Medici plant research 2(3): 47-49
- Kaushik N, Kumar S (2004) *Jatropha curcas* L. Silviculture and Uses. Agrobios (India), Jodhpur
- Kazuo S, Kazuko YS (1996) Molecular responses to drought and cold stress. Curr. Opin.Biotechnol **7**:161–167
- Kingsbury JM (1964) In Poisonous Plants of the United States and Canada. Prentice-Hall Inc., Englewood Cliffs, NJ
- Kreitman RJ, Wilson WH, Robbins D, Margulies I, Stetler-Stevenson M, Waldmann TA, Pastan I (1999) Responses in refractory hairy cell leukemia to a recombinant immunotoxin. Blood 94(10):3340-3348
- Kumar A, Sharma S (2008) An evaluation of multipurpose oil seed crop for industrial uses: A review. Indust Crops Products 28:1-10
- Kumar A, SharmaS (2008) An evaluation of multipurpose oil seed crop for industrial: A review. Indust Crops Products 28:1-10
- Linnaeus C (1753) Species plantarum. In: *Jatropha*. Impensis Laurentii Salvii, Stockholm, pp.1006–1007
- Luo MJ, Liu WX, Yang X, Xu Y, Yan F, Huang P, Chen F (2007) Cloning Expression and Antitumor Activity of Recombinant Protein of Curcin. Russian J Plant Physio 54(2): 202–206
- Mahanta N, Gupta A, Khare SK (2008) Production of protease and lipase by solvent tolerant *Pseudomonas aeruginosa* PseA in solid-state fermentation using *Jatropha curcas* seed cake as substrate. Bioresour. Technol. 99, 1729–1735.
- Makkar HPS and Becker K. (1997) Studies on nutritive potential and toxic constituents of different provenances of *Jatropha curcas*. J. Agric. Food Chem. 45:3152–3157.
- Misra M and Misra AN (2010) *Jatropha*: The Biodiesel Plant Biology, Tissue Culture and Genetic Transformation – A Review *Int. J. Pure Appl. Sci. Technol.*, 1(1), pp. 11-24.
- Mohamed MS *et al.* (2014) Cytological and Subcellular Response of Cells Exposed to the Type-1 RIP Curcin and its Hemocompatibility Analysis 4 : 5747
- Moore K, Greenhut S and Vendrame W (2011) Greenhouse Production of *Jatropha*, a Potential Biofuel Crop

- Mourgue M, Delphaut J, Baret R, Kassab R (1961) Study of the toxicity and localization of toxalbumin (curcin) in the seeds of *Jatropha* curcas Linn. Bull Soc Chim Biol (Paris) 43:517–531
- Mute VM (2009) Anthelmintic effect of *Tamarind indica* linn leaves juice extract on *Pheretima posthuma*. Int J Pharm Res Dev 7:1-6
- Prasad DM , Izam A and Khan M (2012) *Jatropha curcas*: Plant of medical benefits Vol. 6(14), pp. 2691-2699
- Qin W, Xing HM, Ying X, Shen Z. Xu Y and Fang C (2005) Expression of a ribosome inactivating protein (curcin 2) in *Jatropha curcas* is induced by stress. *J. Biosci*.30: 351–357
- Rajanisrosha V, Ananthi T (2013) Physicochemical and Phytochemical Studies On
- Rehm S and Espig G (1992) The cultivated plants of the tropics and sub-tropics. M D Swaine J Trop Ecology 8(1):86 – 86.
- Rug M and Ruppel A (2000) Toxic activities of the plant *Jatropa curcas* against intermediate snail hosts and larvae of schistosomes. Trop Med Int Health 6:423-430
- Sharma US, Sharma UK, Singh A, Sutar N, Singh PJ (2010) In vitro anthelmintic activity of *Murraya koenigii* linn leaves extracts. Int J Pharm Biosci 1(3):1-4
- Shukla A, Singh S and Tiwari S (2015) Transformation of toxic potential of *Jatropha curcas* (Ratanjyot) into protein source: A mini-review 2(2): 89-94
- Solsoloy AD (1995) Pesticidal efficacy of the formulated physic nut, *Jatropha curcas* L. Oil on pests of selected field crops. Philippine J Sci 124:59-74
- Staubmann R, Ncube I, Gubitz GM, Steiner W, Read JS, (1999) Esterase and lipase activity in *Jatropha curcas* L. seeds. J. Biotechnol. 75, 117–126.
- Stirpe F, Battelli MG (2006) Ribosome-inactivating proteins: progress and problems. Cell Mol Life Sci 63:1850–1866
- Stirpe F, Pession-Brizzi A, Lorenzoni E (1976) Studies on the proteins from the seeds of *Croton toglium* and of *Jatropha curcas*. Biochem J 156:1–6 Versuchsw 82:427-30
- Sujatha M (2009) Biotechnological interventions for improving *Jatropha* and Castor for biofuels. petrotech p09-869
- Warra AA (2012) Cosmetic potentials of physic nut (*Jatropha curcas* Linn.) seed oil. ISSN: 2153-649X
- Wink M, Koschmieder C, Sauerwein M and Sporer F (1997) Phorbol esters of *Jatropa curcas*—Biological activities and potential applications. Biofuel and industrial products from *Jatropa curcas*, ed. by Ubitz: 160–166
- Xiaobo Q , Zhang J , Shao C, Lin S, Jiang L, Zhang S, Xu Y and Chen F (2009) Isolation and Characterization of a Curcin Promoter from *Jatropha* curcas L. and Its Regulation of Gene Expression in Transgenic Tobacco Plants. 27:275–281