Establishment of Jatropha (*Jatropha curcas* L.) germplasm and their micropropagation

A

THESIS REPORT

Submitted in partial fulfillment of the
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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled, **"Establishment of Jatropha (*Jatropha curcas* L.) germplasm and their micropropagation"** in partial fulfillment of the requirement for the award of the degree of Master in Biotechnology, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, is an authentic record of my own work during the period of six month from January 2013 to July 2013, under the guidance of Dr. N. Das, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Date: 15 July 2013

Place: PATIALA

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CERTIFICATE

This is to certify that the thesis entitled, **"Establishment of Jatropha (*Jatropha curcas* L.) germplasm and their micropropagation"** submitted by Parika in partial fulfillment of the requirement for the award of the degree of Master of Science in Biotechnology, to Thapar University, Patiala is a record of student's own work carried out by her under my supervision and guidance. The thesis has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

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ABSTRACT

Jatropha curcas L. is a commercially important non-edible oilseed crop known for its use as an alternate source of biodiesel. Presently, bio-fuels are being considered as renewable source of energy derived from various biological raw materials. *Jatropha* species are primarily propagated through seeds; therefore, they are heterozygous in nature. Based on the published reports, it is now known that there is a considerable variation in terms of seed yield and oil content between the different *Jatropha* species available globally. Usually, oil content varies from 10 - 55 %. It is perennial crop, and proper fruit/seed settings require 3 to 4 years time. The seed viability and rate of germination are low, and quality seed screening is another laborious task. Therefore, propagation of this plant through seed alone cannot provide quality planting material for sustainable production of biodiesel from this crop. To ensure adequate biodiesel production, major challenges include a) findings of elite *Jatropha* germplasm through survey and selection, b) to produce large number planting materials in a relatively short period of time. Therefore, plant tissue culture techniques using *Jatropha* germplasm are quite promising. Moreover, such types of techniques are also prerequisite to undertake plant transgenic to produce designer crops with desirable phenotypes. In this context, this project work is quite relevant. In the present investigation, a few plant tissue culture techniques were adopted for establishment of the *Jatropha* germplasm in the laboratory. First, using suitable media formulations efforts were made to establish embryo culture from few *Jatropha* accessions. Embryo raised plantlets plantlets were further used for clonal propagation and the growth characteristics were noted. In other attempt, vegetative parts like shoot tip and nodal segments were excised from the field-grown *Jatropha* plants. After proper surface sterilization, the explants were allowed to grow aseptically on Murashige and Skoog's medium supplemented with various growth regulators for the purpose of multiple shoot induction. Multiple shoot induction was effected from shoot apices and nodal explants on MS medium supplemented with various growth regulators in different combinations. Multiple shoot induction occurred on MS supplemented with BAP (0.5 - 4 mg/l) and kinetin (0.5 - 4 mg/l) either alone or in combination with IAA (0.5 -1 mg/l). Multiple shoot induction from *in vitro* embryo culture raised plantlets was effectively initiated on MS medium supplemented with BAP (1 mg/l) + IAA (0.5 mg/l) with an intervening callus phase. Multiple shoot proliferation from shoot tips taken from young field grown plants was best induced on MS medium supplemented with only BAP (1 mg/l). Similarly from *in vivo* raised nodal segments, initiation of multiple shoot proliferation was observed on MS medium supplemented with BAP (1 mg/l). In conclusion, this study provides useful information for induction of multiple shoot proliferation with the use of different media formulations.

ABBREVIATIONS

MS	Basal Murashige and Skoog's medium
BAP	Benzylaminopurine
BA	N^6 benzyladenin
Kn	Kinetin (6- Furfuryl amino purine)
IAA	Indole-3-acetic acid
IBA	Indole-3- butyric acid
NAA	α-naphthalene acetic acid
TDZ	Thidiazuron
PGRs	Plant growth regulators
PPM	Parts per million

CONTENTS

Page No.

1. INTRODUCTION

Ever increasing global demand for energy, the fast depletion of fossil fuel reserves and concern over climate change have given a pathway to a resurgence in the development of alternative sources of energy. Moreover, it is agreed worldwide that there is need to develop sustainable alternative sources of energy in the coming years. Bio-fuels and bio-energy provides a wide range of alternative sources of energy of biological origin. Presently, bio-fuels are being considered as renewable source of energy derived from various biological raw materials. Two important sources of bio-fuels are ethanol and bio-diesel which are rapidly gaining worldwide acceptance as one of the solutions for problems of environmental degradation, energy security, restricting imports, rural employment, agricultural economy, owing to reduced dependence on oil import; saving in foreign exchange and reduced vehicular pollution. Particularly, in the transport sector, bio-ethanol and bio-diesel are becoming popular in many countries across the world. Due to day to day increase in the consumption of petroleum products in the transport sector, the Indian National Bio-fuel Policy aims at blending 20% bio-ethanol and biodiesel with gasoline and diesel, respectively by 2017.

Recently, Government of India launched national mission on biodiesel with a view to find cheap and renewable liquid fuel based on vegetable oils. However, shortage of raw material to produce biodiesel is a major constraint. Many developed countries are using edible oil-seed crops such as Sunflower and rape-seed in Europe, soybean in the USA, palm oil in Malaysia and coconut in Philippines for the production of biodiesel. However, developing countries like India having shortage of edible oil for consumption cannot afford to use edible oils for production of biodiesel. Nor it can afford to produce more oilseeds by cultivating oil-seed crops in more areas as that implies a shift in cropping pattern in terms of substituting land use from food grains to non food grains. This would cause scarcity of food grains, which in turn will have adverse effects on inflation and poverty. The option therefore left with the country is to go for non-edible oilseeds, which can be grown in degraded forestlands, un-utilised public lands, field boundaries, and fallow lands of farmers where non-edible oil-seeds can be grown (Biswas *et al*. 2010). According to the National Oilseeds and Vegetable

Oils Development Board of the Indian Ministry of Agriculture (NOVOD), there are many oil producing crops and plants with economic potential for biodiesel production. Out of which, *Jatropha curcas* have evoked huge interest all over the tropics as potential biofuel plant. Since, *Jatropha curcas* does not compete with conventional crops for cultivation, the dilemma of food verses fuel does not arise (Ghosh *et al*., 2007).

1.1. ***Jatropha curcas:*** **characteristics of the biodiesel plant**

Jatropha curcas L. (family *Euphorbiaceae)* is native plant of Central America, but is now found abundantly in many tropical and subtropical regions throughout the Africa and Asia because of likely distribution by Portuguese ships via Cape Verde islands. *Jatropha curcus i*s locally known by various names among different states all across the country such as ratanjot, jamalghota, safed arind, parvata-randa, bagbhe- renda, jangliarandi, purging nut or jamlota. Among the various *Jatropha* species *Jatropha curcas, J.glandulifera, J.gossypifolia, J.integerrima, J.multifida, J.nana, J.podagrica* and *J.tanjorensis* are widely cultivated, naturalized and distributed in India.

1.1.1. Plant Morphology

Jatropha is a perennial, deciduous and diploid (2n = 22) small trees or large shrub which can attain a height of 3 to 5 m, but can reach a height of 8-10 m under favourable conditions. Trunk is straight, branching low above the ground; bark is thin and yellowish. Leaves are 6 x 15 cm and three-to five-lobed. The plant is monoecious and flowers are unisexual; occasionally hermaphrodite flowers occur. Flowering occurs during the wet season often with two flowering peaks i.e., during summer and autumn. Fruits are grey-brown capsule, up to 4 cm long; it is normally divided into 3 cells, each containing one seed. Rarely four seeds have also been observed but it is an anomaly and cannot be considered as a trait for improvement. Seeds are black, about 2 cm long and 1 cm thick. The seeds contain 21% saturated fatty acids and 79% unsaturated fatty acids and they yield 25-40% oil by weight. Additionally, the seeds contain other chemical compounds such as saccharose, raffinose, stachyose, glucose, fructose, galactose and protein. *Jatropha* contains curcasin, arachidic, linoleic, myristic, oleic, palmitic and stearic acids. Curcin and phorbol ester are toxic compounds contained in *Jatropha* meal

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1.1.2. Plant adaptability

Because of its drought-resistant nature the species is well adapted to arid and semi-arid conditions. The current distribution shows that introduction has been most successful in the drier regions of the tropics with annual rainfall of 300-1000 mm. It occurs mainly at lower altitudes (0-500 m) in areas with average annual temperatures well above 20°C but can grow at higher altitudes and tolerate slight frost. *Jatropha* grows on well-drained soils with low nutrient content. The *Jatropha* can grow on lands which are not suitable for agriculture purposes and it leads to both improvement of the environment and supply raw material in the form of seeds for local farmer communities at village level. Its leaves and stems are toxic to animals. Being rich in nitrogen, the seed cake is also an excellent source of plant nutrient. The species is widely grown in the tropics as living fences because it is easily propagated by cuttings and not browsed by cattle.

1.2. Importance of *Jatropha curcas* seeds

The seed of *Jatropha curcas* contains 30-45% oil which is used as an insecticide for soap production and numerous other purposes. The seed oil can also be used as a substitute for diesel oil in engines and in recent years special interest has been shown in the cultivation of *Jatropha* in energy plantations. The seed cake obtained after oil extraction have potential applications as fertilizer/organic manure. It has nitrogen content similar to chicken manure (3.2-3.8%). The seeds are not edible mainly due to a high content of toxic principles like curcin and phorbol esters but all parts of the plant are used in traditional medicine. However, some provenances have been reported to produce edible seeds and in Mexico; the seeds from a non-toxic variety are eaten after roasting. Being drought tolerant, it can be used to reclaim eroded areas. Unfortunately it is host for the cassava virus that can be transmitted to the crops and it should never be used for fences around cassava fields. It is also prone to other virus attacks when planted in humid or crowded conditions. With the combination of oil production and erosion control and the ability to grow in marginal areas with poor soil and low rainfall, this species has great potential in rural development as a source of household income and at the same time creating environmental benefits. The oil has a strong purgative action and is also widely used for skin diseases and to soothe rheumatic pain. A decoction of leaves is used against cough and as an antiseptic after birth. As a second-generation non edible biofuel crop, it can affordably and sustainably help to provide a portion of the current fuel supply with minimal environmental impact. The goal of second-generation biofuel is to increase the biofuel supply with crops such as *Jatropha*, castor (*Ricinus communis*) and Camelina (*Camelina sativa*). Among all the oil seed bearing plants, *Jatropha* yields a considerable amount of nonedible oil that can be converted to biodiesel.

1.3. Prospects of *Jatropha Curcas as* biodiesel crop in India

India relies heavily on crude oil imports, and this trend will continue due to the rapid growth of its economy and population. In order to foster energy security, India's strategy is to focus efforts toward energy self-reliance and developing renewable energy options. India proposed an indicative bio-fuel blending target of 20 percent for both bio-ethanol and biodiesel by 2017 (B20 target). Besides fostering India's energy security and combating climate change, another main driver was to increase the productivity of the estimated 55 million hectares of marginal land in India and thus, provide additional employment to the vast rural population. *Jatropha curcas* was identified by the Indian government as one of the most suitable biodiesel feed stocks, since it is able to grow on marginal land and yields high-quality oil suitable for energetic use. India set an ambitious target of 11.2–13.4 million hectares to be planted with *J. curcas* by 2012. Several studies have underlined that using *J. curcas* biodiesel reduces greenhouse gas (GHG) emissions by about 8 to 88 percent compared to the use of fossil diesel (Gmundar *et al*,2012).

1.4. Biotechnological aspects of *Jatropha*

Jatropha curcas is a wild crop. Before it was recognized as a source of Biodiesel, focus had never been on oil production. Moreover, there is lack of benchmark descriptions regarding genetic variability and scientific evidences for elite accessions.

Genetic improvement of *Jatropha curcas* by transformation is required to develop varieties having desirable characters like high seed yield and oil content, resistance to pests and adaptability to different agro-climatic condition. Present trend focuses on selection of plants showing positive traits and improving them by conventional and molecular breeding and large scale vegetative propagation.

Plant tissue culture techniques are used by plant propagators to achieve elimination of viruses from infected plants, rapid multiplication of clones, vegetative propagation of species which are difficult to propagate by other means, all the year round propagation of clones, rapid multiplication of seedlings.

In India *Jatropha curcas* is primarily propagated through seed-raised plants which have various drawbacks like: a) It is difficult to maintain the genetic quality of seeds in subsequent generations. b) Significant variation in seed yield and oil content has been observed in seed raised plants (Pant *et al*., 2006; Jha *et al*., 2007). c) Plants propagated through seeds show heterozygosity and variable seed oil content from 4 to 40%. d) Due to its perennial nature, seed setting requires 2 to 3 years time. e) The seed viability and rate of germination are low, and quality seed screening is another laborious task, thus seed propagation alone cannot provide quality planting material for sustainable agriculture. *Jatropha* plant propagation can also be carried out through stem cuttings. However, there are limitations in this case also like: a) availability of sufficient amount of quality planting material b) plant propagation through cuttings is season based. Thus, conventional propagation through seeds in not reliable and vegetative propagation through stem cuttings is not sufficient to meet the demand (Heller, 1996; Openshaw, 2000). Therefore, *Jatropha* improvement programmes by current methods of agro-biotechnology are of interest all across the world. This has increased the importance of tissue culture technique to allow large scale production of true to type plants and for the improvements of this species through application of genetic engineering techniques.

In the recent years, tissue culture has emerged as a promising technique to obtain genetically pure elite populations under *in vitro* conditions. *In vitro* propagation also known as micropropagation is infact the miniature version of conventional propagation, which is carried out under aseptic conditions. The advent of *in vitro* tissue culture technique has provided a new way to carry out morphogenetic investigations. It facilitates a living system to be studied under controlled environmental conditions. This also enables a study of complex biological phenomenon in parts. Moreover, these partial processes are amenable to controlled investigations.

Plants raised through micropropagation are:

- Of uniform quality
- Pathogen free
- Can be produced much more rapidly a new cultivars could become commercially available within 2 to 3 years from development rather than 5 to 10 years needed using conventional propagation
- Produce uniformly superior seeds
- Show improved vigor and quality

Methods for micropropagation of plants under *in vitro* condition:

- ➢ Multiplication through growth and proliferation of existing apical shoots excised from the parent plant
- ➢ Multiplication through induction of adventitious shoots of existing meristems within axillary buds which proliferate after removal from the parent plant
- ➢ Multiplication through calli raised from different organs and tissues and their subsequent subculturing leading to organogenesis or somatic embryogenesis
- ➢ Multiplication through direct induction of shoots on the explants

2. REVIEW OF LITERATURE

The following sections deals with existing knowledge on *Jatropha curcas* with respect plant tissue culture studies as available in the literature. The last decade witnessed a blooming interest in the development of *in-vitro* culturing technology for the energy crop *Jatropha curcas.*

2.1. Bio-diesel plant- *Jatropha curcas L.*

The plant has been attracting considerable attention as an alternative feedstock for bio-diesel production. The recognition that *Jatropha* oil can yield high quality bio-diesel has led to a surge of interest in this species across the globe. Due to gradual depletion of world petroleum reserves and the impact of environmental pollution, there is urgent need for suitable alternative fuels for use in diesel engines. *Jatropha curcas L.* a non-edible seed bearing plant grows under a variety of agro-climatic conditions and is found in most of the tropical and subtropical regions of the world. Now, it has been recognized as new energy crop for the countries to grow their own renewable energy source with many promising benefits (W. Parawira, 2010).

The *Jatropha* seeds contain about 30-35% oil that can be converted into biodiesel by a process called trans-esterification in which simple alcohol (methanol/ethanol) replaces glycerol from the vegetable oil molecules. The process uses an alkali Potassium or sodium hydroxide (KOH or NaoH) as catalyst. (Hirata *et al.,* 2007; Padhi, 2009; J. Folaranmi, 2012*).* Most research on *Jatropha curcas* for bio-fuel production focused on mechanized plantation agriculture. According to FACT-ADPP *Jatropha* project in Cabo Delgado, Nothern Mozambique, on a small scale instead of converting the oil to bio-diesel, engines are converted to run *on Jatropha* oil (Nielsen *et al*., 2009).

2.2. Plant tissue culture of *Jatropha curcas L.*

In-vitro plant tissue culture is the culturing and maintenance of plant cells or organs in sterile, nutritionally and environmentally supportive conditions. In commercial settings, tissue culture is primarily used for plant propagation and is often referred to as Micropropagation (George *et al*, 2008). Plant tissue culture now has direct commercial applications as well as value in basic research into cell biology, genetics and biochemistry. Researchers had tried to encounter the problem of yield of specific phyto-constituents which are pharmacologically useful in *Jatropha curcas*. Several efforts have been made to establish protocols for micropropagation and regeneration from different explants of *Jatropha. In-vitro* culture exploits flexibility of plant cells and depending on inherent and induced competence of plant cells, systems of plant regeneration are categorized as direct and indirect regeneration.

2.2.1. Induction of callus

Callus induction has been achieved from various parts of the plant, i.e., hypocotyls, cotyledon, leaf, petiole and stem (Batra *et al*. 2007; Kumar *et al*. 2008). Callus induction was induced from leaf explants under high concentrations of BA (5.0 mg/l) with NAA (1.0 mg/l) within 3-4 weeks (Rajore and Batra, 2007). Interestingly, NAA (1.0 - 4.0 mg/l) alone in half MS also induce callus. This finding suggests that *Jatropha curcas* is highly sensitive to auxins with respect to the stimulation of cell division rather the induction of roots (Shrivastava *et al*., 2008). Replacing NAA by IBA in combination with BA also leads to induction of callus (Deore *et al*, 2008).

According to Varshney *et al. (*2010) the immature embryo cultured on different combinations of auxins and cytokinins showed a good response of callus induction (85.7%) and subsequent plant regeneration (70%) with high number of plantlets (4.7/explants) on MS medium supplemented with IBA (0.5 mg/l) and BA (1.0 mg/l).

2.2.2. Shoot bud culture

According to Datta *et al*. (2008), the axillary shoot bud proliferation from nodal explants was best initiated on MS basal medium supplemented with 22.2 µM (BA) and 55.6 µM adenine sulphate, in which cultures produced 6.2 + 0.56 shoots per nodal explants with 2.0 + 0.18 cm average shoot length after 4-6 weeks. The shoot multiplication and root induction was enhanced by using various growth regulators. Then, plantlets were successfully acclimatized in soil with 87% survival frequency. Basu *et al.* (2010) developed an efficient and reproducible *in vitro* plant regeneration system from shoot apices of *Jatropha curcas*.

2.2.3. Nodal stem segment culture

Most frequently used explants for the induction of multiple shoots is the nodal stem segments. The efficient regeneration method via axillary nodes for *Jatropha curcas* was achieved on MS medium with BA (3.0 mg/L) + IBA (1.0 mg/L) + Adenine sulfate (25mg/L) + Glutamine(50mg/L) + L-arginine (15 mg/L) + Citric acid (25 mg/L) by S. Shrivastava and M. Banerjee (2008). They propagates about 10 shoots/axillary node in 3-4 weeks. Singh *et al* (2009) produced 10-15 shoots per axillary node on MS medium supplemented with 1.0 mg/l BA in combination with 1.0 mg/l Kn.

Jatropha plantlets generated by axillary bud proliferation (micropropagation) using nodal segments obtained from selected high yielding genotypes were assessed for their genetic stability using Randomly Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) analysis. By micropropagation plantlets were obtained from the 2nd sub-culture, 4 out of a total of 177 bands scored were polymorphic, but in the 8th and 16th sub-cultures (culture cycle) no polymorphisms were detected. AFLP analysis revealed 0.63%, 0% and 0% polymorphism in the 2nd, 8th and 16th generations, respectively (Sharma *et al*., 2011).

2.2.4. Leaf and petiole culture

The major advantage of using leaf explants is that it has greater multiplication potential. This is because the nodal segment propagation is limited to the number of axillary buds whereas leaf explants may induce a multitude of new shoots, depending on the regeneration capacity.

Deore and Johnson in 2008 gave the protocol for induction of adventitious shoot buds and plant regeneration from leaf disc cultures of *Jatropha curcas*. They germinated the leaf explants on MS medium supplemented with 22.7 µM TDZ, 2.22 µM BA and 0.49 µM IBA. They concluded that TDZ and high cytokinin is responsible for greater adventitious shoot bud induction where as BA in the absence of TDZ promoted the callus induction.

Leaf explants of *Jatropha curcas* cultured on MS medium supplemented with 0.98 µM thidiazuron (TDZ) in combination with 0.98 µM IBA produced direct shoot buds and also high copper content enhance the shoot bud induction by 10 times on MS medium supplemented with 2.46 µM IBA (Kothari *et al*.,2010).

Kumar *et al*. (2010) reported the shoot regeneration through direct organogenesis from cotyledonary leaf explants using MS medium supplemented with different concentrations of TDZ and other growth regulators. The elongated shoot treated with 15 µM IBA, 5.7 µM IAA and 11 µM NAA resulted in highest percent rooting and the rooted plants could be established with 90% survival rate in soil.

Reddy *et al*. (2009) developed an efficient and reproducible protocol for the regeneration of *Jatropha curcas* from petiole explants without the formation of intervening callus using MS medium supplemented with 2.27 µM TDZ. They reported 58.3% shoot bud induction and 10 shoots per explants were obtained.

2.2.5. Somatic embryogenesis and embryo culture

Astha *et al*., 2006 developed a regeneration protocol from embryo cultures. Somatic embryos were cultured on MS medium supplemented with different concentrations of NAA, IAA, IBA and 2, 4-D in combination with Zeatin. Induction of globular somatic embryos in 58% of the incubated cultures was achieved on MS basal medium supplemented with different concentrations of Kinetin in combination with IBA.

Somatic embryogenesis in *Jatropha curcas* leads to the establishment of efficient plant regeneration by using leaf explants. The induction of globular somatic embryos in *Jatropha curcas* was achieved on MS medium with 2.3 µM Kinetin and 1.0 µM IBA along with 13.6 µM adenine sulphate. It was most effective for somatic embryo induction (80%) in *Jatropha curcas* (Jha *et al.,* 2007). Recently Banerjee *et al.* (2009) showed direct organogenesis from *Jatropha curcas* embryo cultures.

2.3. Genetic transformation and tissue culture

To avoid the disadvantages like low productivity and susceptibility to pests in *Jatropha curcas*, varietal improvement by genetic engineering is essential. *Agrobacterium* (Ag) mediated transformation is the main method used for developing transgenic plants. The choice of explants having competence for transformation and regeneration is a crucial factor in micropropagation.

Li *et al*. (2006) were the first to report genetic transformation from callus culture of *Jatropha curcas* through *Agrobacterium*-mediated transformation. The overall transformation efficiency was 13%. To enhance the transformation efficiency of *Jatropha curcas* physical wounding-assisted (i.e., sonication and sand-vortexing in addition to shaking) *Agrobacterium*-mediated transformation was reported which demonstrate that physical wounding was able to efficiently ameliorate the transformation efficiency of juvenile cotyledons of *Jatropha curcas* (Khemkladngoen *et al.* 2011). Embryogenic cell cultures at different developmental stages were co-cultivated with *Agrobacterium* to determine the best stage of transformation on *vitis spp.* which concluded that proembryonal masses and cotyledonary stage of embryos were best for transformation of *V.rotundifolia, V.rupestris* and certain *Vitis hybrids (*Dhekney *et.al*). A gene transfer system that ensured recovery of whole plant transformants was developed from safflower cotyledons through *Agrobacterium*-mediated transformation and PCR amplification of uidA and nptII marker genes were used for early determination of putative transformants (Rohini V.K. and Rao K. Sankara , 2000).

2.4. Toxicity of *Jatropha curcas* seeds

Jatropha kernel meal is very high in protein. The kernel meal left over from oil extraction has a protein around 65% of dry matter. This protein is toxic to animals due to the presence of curcin and phorbol esters. While curcin can be neutralized by heat treatment, phorbol esters look to present more of processing challenge. They are not carcinogenic but have great capability to amplify the effect of a carcinogen. The seeds of *Jatropha curcas* reported to be orally toxic to living organisms and phorbol esters have been identified as the main toxic agent.

Adolf *et al.* (1984) were the first to identify phorbol esters in *Jatropha curcas,* but they could not elucidate their exact chemical structure. To remove the phorbol esters from the seeds of *Jatropha* - biological, physical and some chemical detoxification methods of *Jatropha* seed have been proposed but still research on detoxification mechanisms is necessary (Wakandigara *et al*.,2013).

Latex of *Jatropha* is used as herbal medicine which is traditionally used as dental pain relief. Farida *et al*. (2012) worked to detect the cytotoxicity of extracted latex to epithelial and fibroblast cells which concluded that *Jatropha curcas* was toxic to these cells. In Mexico, two genotypes of *Jatropha curcas* are available; the toxic and non-toxic types. The seeds of non-toxic type are consumed by humans after roasting (Heller, 1996).

Jatropower AG, a Swiss domiciled company was founded in 2007. It has established a research which leads in the study and development of non-toxic genotypes of *Jatropha curcas* from Mexico. They also offer elite accessions of non-toxic *Jatropha* planting material which is situated at Switzerland.

2.5. Commercial uses of *Jatropha curcas*

Jatropha is generally grown as live fence for protection of agricultural fields against damage by livestock. The glycerin which is a by-product of bio-diesel produced from *Jatropha* oil can be used to make soap (Kumar and Sharma 2008).

It can be used as pesticide. Substances such as phorbol esters, which are toxic to humans and animals, have been isolated and their molluscicidal, insecticidal and fungicidal properties have been demonstrated (Solsoloy *et al*., 1997).

All parts of *Jatropha* like seeds, leaves and bark have been used in traditional medicine and for veterinary purposes for a long time (Duke *et al*., 1988).The latex of *Jatropha* contains several alkaloids like Jatrophine, Jatropham and curcain with anti cancer properties (Thomas *et al*., 2008).The roots are reported as an antidote for snake-bites. The anti-inflammatory activity of *Jatropha curcas* L. root is reported by Mujumdar and Misra (2004). Curcin, a protein isolated from the seeds of *Jatropha* can be used as cell-killing agent to elaborate the purification methods and investigate the anti-tumor activity (Luo *et al*., 2006).

Seed cake is a by-product of oil extraction which contains curcin, a highly toxic protein similar to ricin in castor, making it unsuitable for animal feed. However it does have potential as a fertilizer or biogas production (Gubitz *et al*., 1999), if available in large quantities. The seed cake is also rich in nitrogen content ranging from 3.2 to 3.8% which can replace the use of chemical fertilizer (Kumar and Sharma 2008). The experiments done on solid-state fermentation of *Jatropha* seed cake showed that, it could be a good source of low cost production of industrial enzymes (Mahanta *et al*., 2008).

3. Aim of the present study

The aim of the present study was to adopt and standardize different media formulations for establishment of Jatropha (*Jatropha curcas* L.) germplasm and subsequently their micropropagation under *in vitro* conditions using different plant parts like embryos, shoot tips and nodal explants from high-yielding elite clones. Such types of techniques are helpful for large scale production of quality planting material in a short time and limited laboratory space. This work involved only a few elite *Jatropha* accessions collected from the state of Punjab. Keeping this in view the following objectives were framed:

4. OBJECTIVES

- To adopt/modify protocols for raising *Jatropha* plantlets through embryo culture techniques
- To adopt/modify protocols for micropropagation using shoot tip and nodal explants of field-grown *Jatropha* plants
- To see kanamycin sensitivity of the *Jatropha* plantlets

5. MATERIAL AND METHODS

5.1.Materials

5.1.1. Plant material and source of explants

The plant material used in tissue culture needs to be healthy and actively growing. Seeds from elite *Jatropha* clones for development of embryo culture under aseptic conditions were collected from the different accessions growing in the different regions in the state of Punjab. For micropropagation the best performing *Jatropha curcas* genotypes (TJS-01#04, TJS-04 #42) identified in the field conditions on the basis of seed yield and seed oil content were selected for *in-vitro* aseptic culture of younger and fresh nodal explants and shoot tips.

5.1.2. Chemicals

The routinely used chemicals were purchased from Hi-media Laboratories Pvt. Ltd., Mumbai and Sisco Research Laboratories Pvt. Ltd., Mumbai.

5.1.3. Glass wares

Glass wares and Plastic wares were purchased from Borosil and Tarsons Products Pvt. Ltd. The glassware used for experimental work comprised of 100 ml, 150 ml, 250 ml, 500 ml and 1000 ml of conical flasks and culture bottles (8×3 inches). In addition, other glassware included measuring cylinders (100 ml, 500 ml) and beakers. Before usage, glassware were washed with alkaline detergent teepol and then cleaned with running tap water. These were then thoroughly brushed with chromic acid solution (mixture of $K_2Cr_4O_7 + H_2SO_4 + H_2O$) followed by thorough washing with tap water. The glassware was then inverted in a clean tray and left to dry in the oven.

5.1.4. Other materials

Laminar air flow hood, ethanol, oven, plant tissue culture room, autoclave, cotton, detergent etc.

5.1.5. Culture Medium

The media formulation described as Murashige and Skoog's (1962) referred as MS medium (Table 1) was selected as the optimal culture medium. The significant feature of the MS medium is its very high concentration of nitrate, potassium and ammonia salts. Stock solutions of major elements, minor elements and vitamins were prepared and stored in a freeze chest at 4º C. These stocks were used in desired proportions only before use. The different phyto-hormones were used either singly or in combination for the induction of multiple shoot formation.

Table 1: Composition and Stock Preparations for Murashige and Skoog (MS) Basal Medium:

1. MS Major Salts

S.No.	MS Major Salts	MS Basal conc. (mg/l)	Amount required for 100X stock (g/l)	Use of stock for 1L medium (ml)
1.	KNO_3	1900.0	190.0	10.0
2.	NH_4NO_3	1650.0	165.0	10.0
3.	$MgSO_4.7H_20$	370.0	37.0	10.0
4.	$CaCl_2.2H_2O$	440.0	44.0	10.0
5.	KH_2PO_4	170.0	17.0	10.0

Note: All the MS major salts stock solutions to be prepared separately.

2. MS Minor Salts

S.No.	MS Minor Salts	MS Basal conc. (mg/l)	Amount required for 1000X stock(g/l)	Use of stock for 1L medium (ml)
1.	H_3BO_4	6.20	6.20	1.0
2.	$MnSO_4.4H_2O$	22.30	22.30	1.0
3.	$ZnSO_4.7H_2O$	8.60	8.60	1.0
4.	$Na_2MoO_4.2H_2O$	0.25	0.25	1.0
5.	$CuSO_4.5H_2O$	0.025	0.025	1.0
6.	$CoCl_2.6H_2O$	0.025	0.025	1.0
7.	KI	0.83	0.83	1.0
8.	$Fe_2EDTA. 2H_2O$ (sodium salt)	30	30	1.0

3. MS Vitamins

S.No.	Name of Vitamins	MS Basal conc.(mg/l)	Amount required for 100X stock(g/l)	Use of stock for 1L medium (ml)
1.	Nicotinic Acid	0.5	0.5	1.0
2.	Pyridoxine HCl	0.5	0.5	1.0
3.	Thiamine HCl	0.1	0.1	1.0
4.	Glycine	2.0	2.0	1.0
5.	Myo-inositol	100.0	100.0	1.0

Note: All the MS vitamins stock solutions to be prepared separately.

5.2. Methods

5.2.1. Preparation of Media

The MS medium was prepared by adding all the components in required amounts as given in the Table 1. All the constituents except agar were mixed included 2.5% sucrose as carbon source and then the pH of the solution was adjusted to 5.5 to 5.8. Later, agar (0.7%) was added and the medium was heated to boil so as to homogenize agar. After cooling, definite aliquots of the medium were then poured into sterile bottles under aseptic conditions. After pouring in to bottles, media was autoclaved at 121 ^{0}C for 20 minutes at 15 lb/in^2.

Following are some of the supplements which were used either singly or in combination for the differentiation and multiple shoot formation from nodal and shoot tips segments of *Jatropha curcas*.

a) MS Basal Medium (MS)
b) MS + BAP (1mg/l) + IAA (0.5mg/l) = M1
c) MS + BAP (2mg/l) + IAA (0.5mg/l) = M2
d) MS + BAP (1mg/l) + IAA (1mg/l) = M3
e) MS + BAP (2mg/l) + IAA (1mg/l) = M4
f) MS + BAP (3mg/l) + IAA (1mg/l) = M5
g) MS + BAP (4mg/l) + IAA (1mg/l) = M6

h) MS + BAP (1mg/l) = M7
i) MS + BAP (2mg/l) = M8
j) MS + BAP (3mg/l) = M9
k) MS + BAP (4mg/l) = M10
l) MS + Kinetin (1mg/l) = M11
m) MS + Kinetin (2mg/l) = M12
n) MS + Kinetin (3mg/l) = M13
o) MS + Kinetin (4mg/l) = M14
p) MS + IAA (0.5mg/l) = M15
q) MS + IAA (1mg/l) = M16
r) MS + Kanamycin (50 mg/l) = M17
s) MS + Kanamycin (100 mg/l) = M18

After autoclaving, media was left to cool down by keeping it at room temperature. Then, growth regulators were added under laminar air flow hood in appropriate amounts into the tissue culture bottles. Bottles were left for 1-2 hrs to solidify the media. Media was used after two days by observing any bacterial or fungal contamination.

5.2.2. Maintenance of Aseptic conditions

All the experimental manipulations were carried out under strictly aseptic conditions in laminar air flow bench fitted with a bactericidal U. V. tube (15 W, peak emission 2637 Å). The floor of the chamber was thoroughly cleaned with alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, scalpels, blade etc.), gas burner, lighter, tube containing absolute alcohol etc were also cleaned with alcohol. The chamber was then sterilized with U.V. rays continuously on for one hour.

5.2.3. Protocol for sterilization of *Jatropha* seed/kernels

Mature *Jatropha* seeds were decorticated to recover kernels in a bottle. *Jatropha* seeds were then imbibed in water for 2-3 hrs. After imbibitions, kernels were treated with 1-2 drops of liquid detergent (Tween-20) for 7-10 minutes. Then, seed kernels were placed under running tap water for 10 minutes to completely remove the detergent. Afterwards kernels were treated with bavistin solution 0.1 - 0.2% for 7-10 minutes. Again the kernels were washed under running tap water for 10 minutes to remove the bavistin. Then, the kernels were treated with

10% sodium hypochlorite solution for 7-8 minutes to kill all the adhering bacterial contamination. After washing under running tap water for 10-15 minutes, further work was carried out laminar air flow chamber. Mercuric Chloride (0.1-0.2%) treatment was then given to *Jatropha* kernels for 5-7 minutes. After that *Jatropha* kernels were given 4-5 sterile water washing to remove excess disinfectant from kernel.

5.2.4. Inoculation of *Jatropha* kernels

Under the sterile conditions *Jatroph*a kernels were carefully placed in the horizontal position on the sterile glass plate, and was given a vertical cut less than half of its thickness with the help of sterile forceps and scalpel and isolated intact embryo. Finally, embryos were inoculated on MS basal medium under aseptic conditions. The bottles were sealed and labeled carefully and were finally kept in the culture room under maintained conditions of temperature (25 ºC) and 16 hours of light regime followed by 8 hours of dark period. The growth of the inoculated explants was monitored regularly after every 7 days.

5.2.5. Inoculation of Nodal explants and Shoot tips

Nodal explants and shoot tips were taken from the 5 years old plant (TJS-01 #04) growing under field conditions of Thapar University, Patiala. These explants were placed in bottles and after covering with net washed for 15 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (Tween-20) for another 3-4 minutes and then washed properly under running tap water for 15 minutes to remove the detergent. The explants were then washed with 0.1-0.2% of bavistin solution for another 3-4 minutes and then washed properly under running tap water for 15 minutes. Hands were wiped with alcohol before inoculation. Instruments (like forceps, scalpels, glass plate etc.) were all sterilized by firstly autoclaving, dipping in the alcohol and flaming a number of times. Care was taken to cool the instruments before putting into operation. The sterilized explants were treated with 0.1-0.2% of mercuric chloride solution for 2-3 minutes to kill bacterial contamination. The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove the traces of $HgCl_2$. Fresh cuts were given to the stem explants after sterilization to remove undesirable or dead portions. These were then planted on variously augmented MS medium. The bottles were sealed and labeled carefully and were finally kept in the culture room under maintained conditions of temperature (25 ºC) and 16 hours of light regime followed by 8 hours of dark period. The growth of the inoculated explants was monitored regularly.

5.2.6. Kanamycin sensitivity test

This test is essential to check the sensitivity of *Jatropha curcas* with regard to antibiotic kanamycin. For this purpose, the seed kernels were taken from the TJS- 04 #42, TJS-27 #108, TJS-34 #01 *Jatropha* accession and were initially sterilized as per previously mentioned protocol 5.2.3. After surface sterilization, the embryos were inoculated on three different media: a) MS basal medium as control b) MS 17 medium (MS + Kanamycin, 50 mg/l) c) MS 18 medium (MS + Kanamycin, 100 mg/l). After the inoculation in laminar air flow chamber the bottles were sealed and labeled carefully and were finally kept in the culture room under maintained conditions of temperature (25 ºC) and 16 hours of light regime followed by 8 hours of dark period. The growth of the inoculated explants was monitored regularly.

6. RESULTS AND DISCUSSION

6.1. Establishments of *Jatropha curcas* germplasm

6.1.1. Embryo culture

To study embryo culture technique, the seeds from few *Jatropha* varieties like; TJS- 04 #42, TJS- 61 #33, TJS- 29 #31, TJS-15 #11, TJS-73 #59, TJS-27 #108 were collected from the plantation sites located in the different areas in the state of Punjab. The collected verities were identified as elite on the basis of seed yield per plant and its oil content. The black *Jatropha* seed coat (Fig. 1) was then removed using mortar and pestle in order to release the white seed kernels (Fig. 2)

Fig. 1 *Jatropha curcas* seeds

Fig. 2 *Jatropha curcas* seed kernels

The Kernels were then sterilized using protocol as mentioned in 5.2.3 and embryos were isolated under aseptic conditions and inoculated on MS basal medium (Fig. 3). Growth was monitored after every week. The radical and plumule part of the inoculated embryos gave rise to roots and shoots respectively. After two weeks of inoculation, the embryos were grown into a healthy plantlet with no bacterial and fungal contamination as shown in (Fig. 4 and Fig. 5).

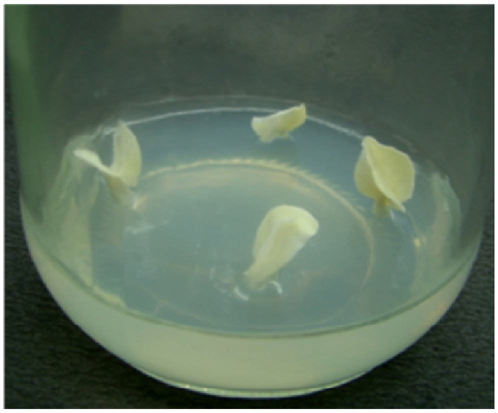


Fig. 3 Embryo inoculation on MS media

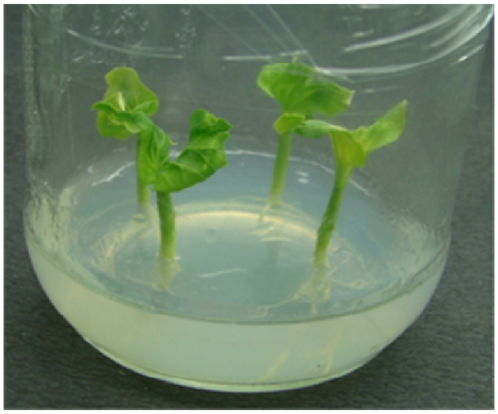


Fig. 4 One week old embryo raised plantlet on MS medium

Fig. 5 Two week old embryo raised plantlet on MS medium

6.1.2. Sensitivity test using antibiotic Kanamycin for embryo culture of *Jatropha*

In the present study, the sensitivity test for antibiotic Kanamycin was carried out on embryo culture raised plantlets of few *Jatropha curcas* accessions *(*TJS- 04 #42, TJS-27 #108, TJS-34 #01). It was observed that, after one week, the control plants (without antibiotic) were growing in healthy conditions with completely green leaves. But, the plantlets were sensitive to both kanamycin 50 mg/l and kanamycin 100 mg/l with yellowing of leaves and poor growth of roots in comparison to control plants in both the concentrations of the drug. It was further observed that kanamycin 100mg/l was more potent than kanamycin 50 mg/l concentration as more yellowing and stunted growth was observed at later concentration. These results showed the inhibitory action of antibiotic kanamycin on the *Jatropha curcas* plant growth (Fig. 6a-c).

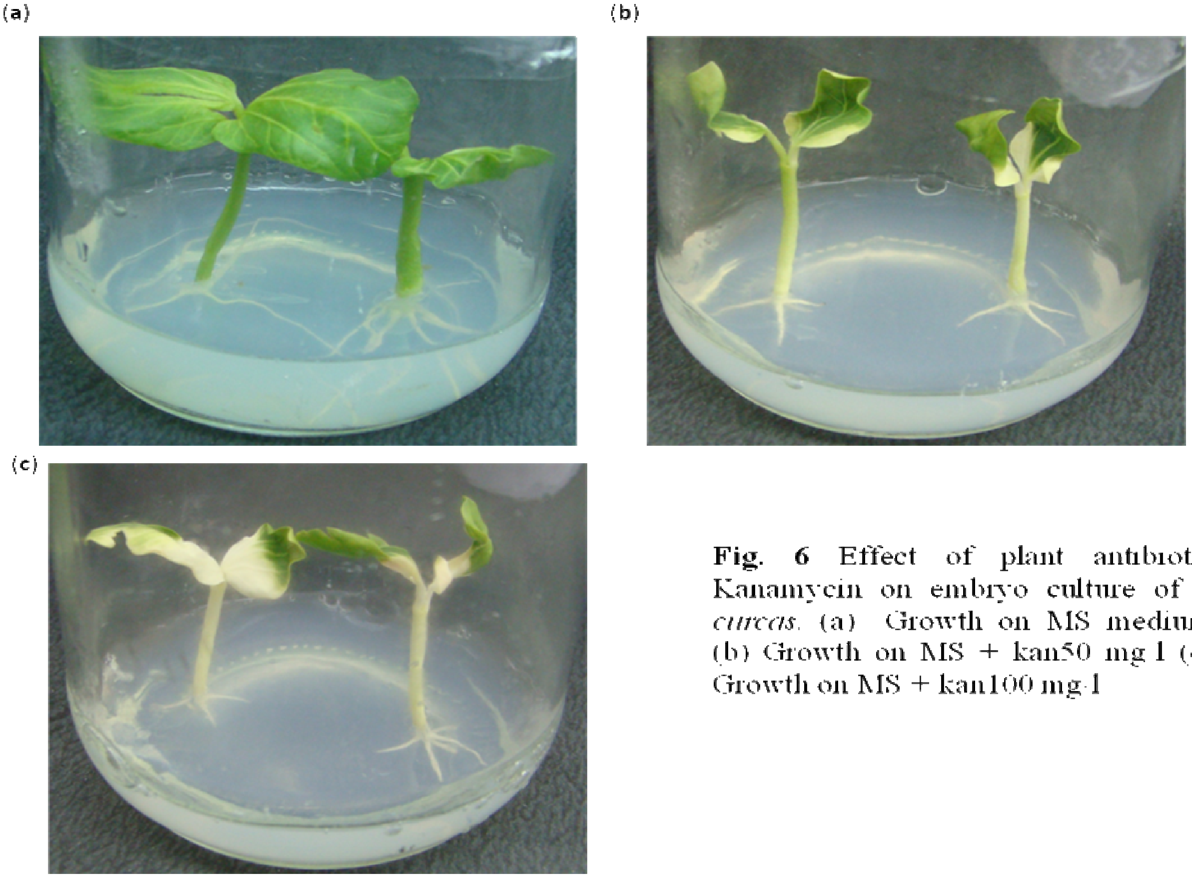


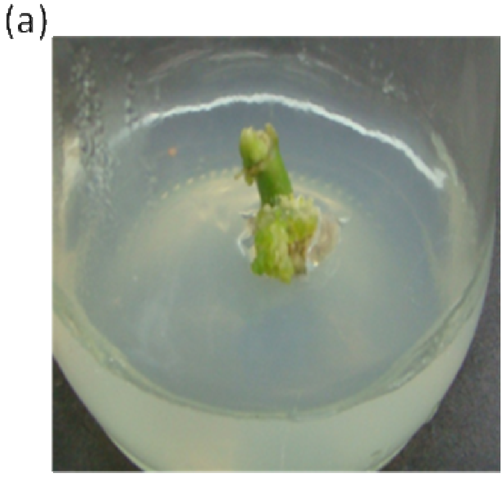
Fig. 6 Effect of plant antibiotic Kanamycin on embryo culture of *j. curcas*. (a) Growth on MS medium (b) Growth on MS + kan50 mg l (c) Growth on MS + kan100 mg l

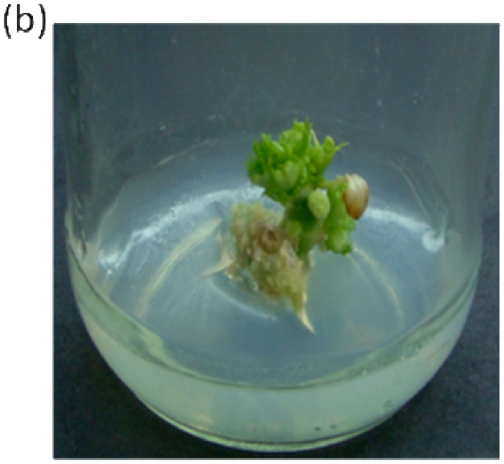
6.1.3 Shoot tip culture from *in vitro* raised plantlets

Access to a prolific system of shoot proliferation from explants with pre-existing meristem is a prerequisite for rapid micropropagation. Shoot apices derived from *in vitro* embryos raised plantlets were found to be ideal owing to their proliferating ability. The responding shoot apices showed visible signs of swelling after 10-15 days and showed the development of shoot buds and callus proliferation at the base of the explants after 15 days on MS medium supplemented with different cytokinins.

Shoot tips were excised from *in vitro* embryo raised plantlet. After surface sterilization shoot tips were cultured on MS medium supplemented with different concentration of growth regulators. Shoot proliferation was induced from shoot tip explant on MS medium supplemented with different concentrations of BAP (0.5 mg/l – 4 mg/l) alone or in combination with IAA (0.5mg/l - 1.0 mg/l). Shoot tips cultured on MS medium supplemented with BAP (1 mg/l) + IAA (0.5 mg/l) (Fig. 6 a-b) induced maximum number of shoots per explant after initial callus induction. Transfer of these shoot buds to lower concentrations of BAP (0.5 mg/l) proved most effective in increasing shoot growth (Fig. 6c). Whereas, higher concentration of BAP (2 mg/l) + IAA (0.5 mg/l) also induced multiple shoots after profuse amount of callus induction (Fig. 7). For the elongation of shoots MS + gibberellic acid ($GA_{3)}$ was added in different concentration (0.5 mg/l – 1 mg/l). The results for shoot elongation are still under progress.

The presence of thidiazuron (TDZ) in the induction medium has greater influence on the induction of adventitious shoot buds, where as BAP in the absence of TDZ promoted callus induction rather than shoot buds (Deore *et al* 2008). Therefore, to avoid intervening callus phase we would also try using other regulators like use of thidiazuron (TDZ) alone or in combination with indole-3-butyric acid (IBA) for adventitious shoot buds formation.





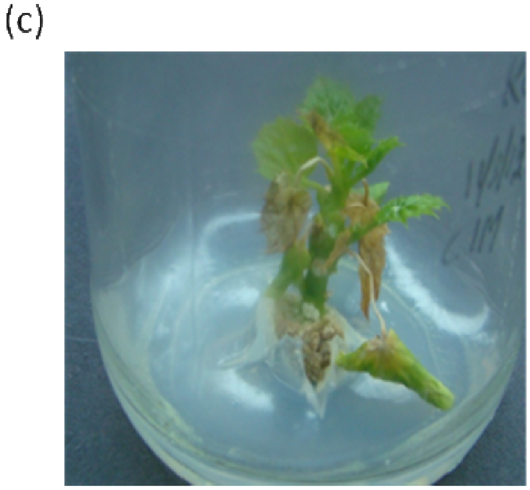


Fig 6: Multiple shoot induction from *in vitro* raised shoot tip explant of *Jatropha curcas* (a) Swollen shoot apex showing the formation of 4-5 adventitous shoot buds on MS + BAP (1 mg/l) + IAA (0.5 mg/l) (b) Initiation of Multiple shoot proliferation on MS + BAP (1 mg/l) + IAA (0.5 mg/l) from shoot apices after initial callus phase (c) Further elongation of shoots on MS + BAP (0.5 mg/l)

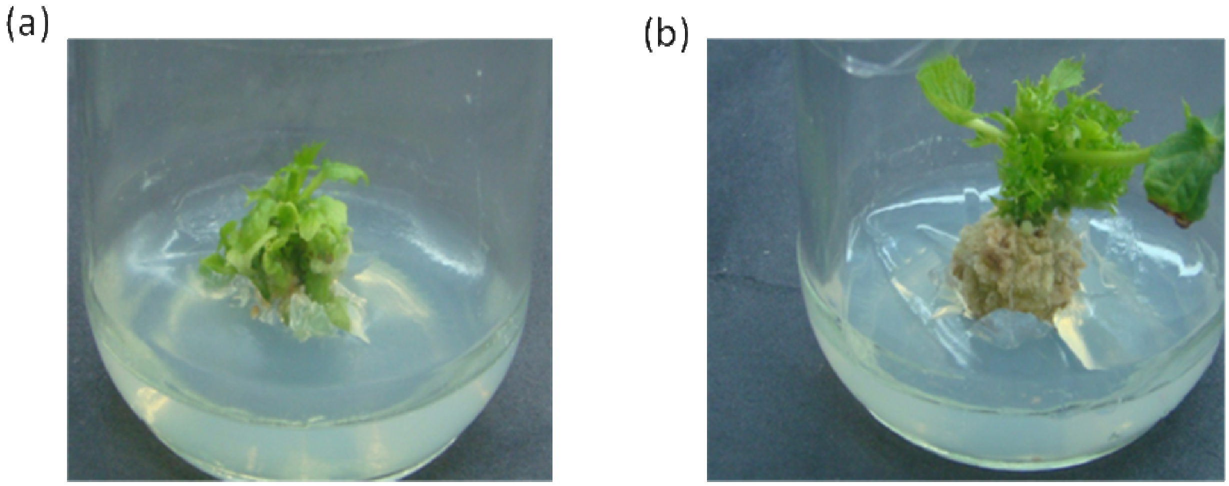
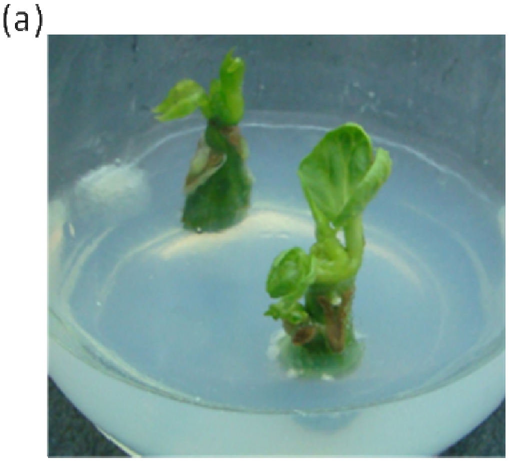


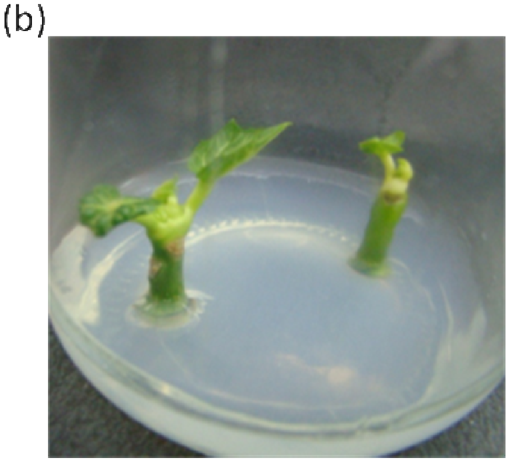
Fig 7: Multiple shoot induction from *in vitro* raised shoot tip explant of *Jaropha curcas* (a) Initiation of Multiple shoot proliferation on MS + BAP (2 mg/l) + IAA (0.5 mg/l) from shoot apices after profuse callus induction (b) Multiple shoot proliferation on BAP (2 mg/l) + IAA (0.5 mg/l) after 4-5 weeks of culturing

6.1.4. Shoot tip culture from field raised plants

Fresh Shoot tips were excised from field grown elite *Jatropha curcas* mature plantlets. They were surface sterilized using protocol as mentioned in 5.2.5. During surface sterilization, the timing of bavistin (fungicide) and mercuric chloride ($HgCl_2$) treatment was very critical because long time exposure to these sterilizing agents leads to browning of the explants. Efforts were made to standardize efficient sterilization protocol for *in vivo* raised shoot tip and nodal explants. Results showed that the treatment of different disinfectants like detergent (3-4 minutes), bavistin (3-4 minutes) and mercuric chloride (2-3 minutes) was sufficient for surface sterilization of soft explants like shoot tips and nodal segments. After surface sterilization, shoot apices were cultured on MS medium supplemented with different concentration of auxins and cytokinins individually at different concentration (0.5 mg/l - 4 mg/l) for multiple shoot induction. Shoot proliferation was induced from shoot tip explant on MS medium supplemented with different concentrations of BAP (0.5 mg/l – 4 mg/l) and kinetin (0.5 mg/l - 4 mg/l) alone or in combination with IAA (0.5 mg/l - 1.0 mg/l). Shoot tips cultured on MS medium supplemented with BAP (1 mg/l) (Fig. 8a) induced maximum number of shoots per explant within 3 weeks of culture. Whereas, shoot tips cultured on MS + BAP (1 mg/l) + IAA (0.5 mg/l) induced multiple shoots with intervening callus induction (Fig. 8b). The frequency of regeneration and number of shoots were lowest on the medium supplemented with 4 mg/l BAP.

Multiple shoot proliferation was also tried on MS medium supplemented with different concentrations of kinetin (0.5 mg/l – 4 mg/l). Shoot tips cultured on MS medium supplemented with kinetin (1 mg/l) (Fig 8C) induced maximum number of shoots per explant without intervening callus induction within 3 weeks of culture. The frequency of multiple shoot induction was very less with higher concentrations of kinetin (4 mg/l). It was also observed that kinetin (1 mg/l) in medium containing BAP (1 mg/l) had no effect on enhancement of multiple shoot proliferation suggesting absence of any synergistic role. The results for shoot elongation on different combinations of medium are still under progress.





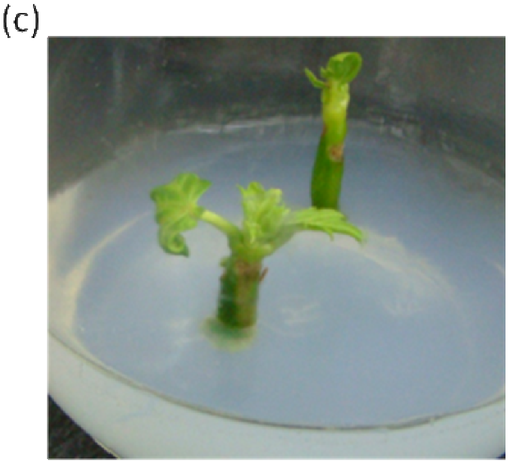


Fig 8: Multiple shoot induction from shoot tip explant of *Jatropha curcas* (a) Initiation of Multiple shoot proliferation on MS + BAP (1 mg/l) from shoot apices (b) Initiation of Multiple shoot proliferation on MS + BAP (1 mg/l) + IAA (0.5 mg/l) from shoot apices with initial callus induction. Further elongation of shoots on MS + BAP (0.5 mg/l) (c) Initiation of Multiple shoot proliferation on MS + kinetin (1 mg/l) from shoot apices

6.1.5 Nodal culture from field raise plants

Nodal explants derived from field grown plants were sterilized using protocol described in 5.2.5. No visible growth was observed on simple MS basal medium even after 4-6 weeks of inoculation. After surface sterilization, fresh cuts were given to the stem explants to remove undesirable or dead portions and nodal segments were cultured on MS medium supplemented with different concentration of cytokinins individually at different concentration (0.5 mg/l - 4 mg/l) for multiple shoot induction. Shoot proliferation was induced from nodal segment explant on MS medium supplemented with different concentrations of BAP (0.5 mg/l – 4 mg/l) and kinetin (0.5 mg/l - 4 mg/l) alone. Best initiation of multiple shoots from nodal segments was observed when cultured on MS medium supplemented with BAP (1 mg/l) (Fig 9A). Nodal segments cultured on MS medium supplemented with kinetin (1 mg/l) (Fig 9B) also induced multiple shoot proliferation within 3 weeks of culturing. No visible growth was observed on simple MS basal medium even after 4-6 weeks of inoculation. Moreover, the frequency of multiple shoot induction was very less with higher concentrations of BAP and kinetin (4.0 mg/l).

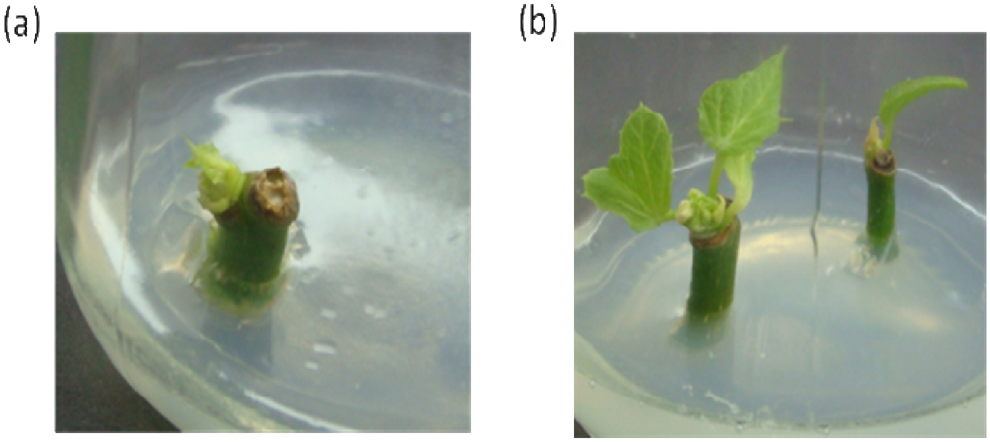


Fig 9: Intiation of Multiple shoot induction from nodal explant of *Jaropha curcas* (a) Initiation of Multiple shoot proliferation on MS + BAP (1 mg/l) (b) Initiation of Multiple shoot proliferation on MS + kinetin (1 mg/l)

Concluding remarks: The present study was a preliminary study to standardize Media formulations for establishment of *Jatropha curcas* germplasm and their micropropagation through tissue culture technique. In the 1st step, we had successfully raised plantlets from mature embryo culture of *Jatropha curcas* under *in vitro* conditions on simple MS medium. Further, sensitivity test for antibiotic Kanamycin was performed on embryo culture raised plantlets of few *Jatropha curcas* accessions. These results indicated sensitivity of *Jatropha curcas* towards antibiotic kanamycin both at concentrations (50 mg/l and 100 mg/l). For micropropagation studies, in the initial step, we had standardized efficient sterilization protocol for *in vivo* raised shoot tips and nodal segments. Multiple shoot induction was best initiated on MS medium supplemented with BAP (1 mg/l) + IAA (0.5 mg/l) from *in vitro* embryo culture raised plantlets with initial callus induction. Multiple shoot proliferation from shoot tips taken from young field grown plants was best induced on MS medium supplemented with only BAP (1 mg/l). Similarly from *in vivo* raised nodal segments initiation of multiple shoot proliferation was observed cultured on MS medium supplemented with BAP (1 mg/l). Similarly, in other result MS medium supplemented with Kinetin (1 mg/l) also leads to initiation multiple shoot initiation from the nodal segments. In conclusion, this study provided us basic information for induction of multiple shoot proliferation with the use of different media formulations. There are few more steps which need to be fulfilled for complete micropropagation protocol like: a) suitable shoot elongation medium b) Root induction medium c) Hardening and acclimatization of micropropagated plants.

7. SUMMARY

Various experimental steps as adopted in the study are briefly discussed below:

- Mature seeds from different accessions of *Jatropha curcas* were surface sterilized using standardized protocol; embryos were excised under sterile conditions and were cultured on MS basal medium. Response was monitored regularly.
- The sensitivity test for antibiotic Kanamycin was carried out on embryo culture raised plantlets of few *Jatropha curcas* accessions. These results showed the inhibitory action of antibiotic kanamycin (both 50 mg/l, 100 mg/l) on the *Jatropha curcas* plant growth. Yellowing of the leaves along with poor root growth was observed for plants raised under kanamycin enriched media.
- Shoot tips were excised from *in vitro* embryo raised plantlets, cultured aseptically on MS media supplemented with various growth regulators and observed the nature of response. Shoot tips cultured on MS medium supplemented with BAP (1 mg/l) + IAA (0.5 mg/l) induced maximum number of shoots per explant after initial callus induction.
- During surface sterilization, the treatment timing for different sterilizing agents like detergent (teepol), bavistin (fungicide) and mercuric chloride ($HgCl_2$) was very critical because long time exposure to these sterilizing agents leads to browning of the explants. Efforts were made to standardize efficient sterilization protocol for *in vivo* raised shoot tip and nodal explants.
- Shoot tips were taken from young field grown plants, sterilized and cultured aseptically on MS media supplemented with various growth regulators and observed the nature of response. Shoot tips cultured on MS medium supplemented with BAP (1 mg/l) induced maximum number of shoots per explant within 3 weeks of culture.
- Nodal segments were taken from young field grown plants, sterilized and cultured aseptically on MS media supplemented with various growth regulators and observed the nature of response. Initiation of multiple shoot proliferation was observed from Nodal segments when cultured on MS medium supplemented with BAP (1 mg/l). Similarly, MS medium supplemented with Kinetin (1 mg/l) also leads to multiple shoot initiation from the nodal segments.

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