Various media formulations for *in vitro* clonal propagation and *Agrobacterium*-mediated genetic transformation of *Jatropha* (*Jatropha curcas* L.)-a promising biodiesel crop

Dissertation

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By

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DECLARATION

I, the undersigned, hereby declare that the research work presented in the M.Tech project entitled "**Various media formulations for *in vitro* clonal propagation and *Agrobacterium*-mediated genetic transformation of Jatropha (*Jatropha curcas* L.)-a promising biodiesel crop.**" is an authentic record of the work carried out by me under the supervision and guidance of Dr. N. Das, Professor, Department of Biotechnology, Thapar University, Patiala.

Further, I declare that no part of this dissertation has been submitted for a degree or any other qualification of any other university or examining body in India/elsewhere.

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CERTIFICATE

This is to certify that dissertation entitled, "**Various media formulations for *in vitro* clonal propagation and *Agrobacterium*-mediated genetic transformation of Jatropha (*Jatropha curcas* L.)-a promising biodiesel crop.**" submitted by **Ms. Shikha Khullar** in partial fulfilment of the requirements for the award of Masters in Technology Degree in Biotechnology at Thapar University, Patiala is an authentic work carried out by her under our supervision and guidance.

To the best of our knowledge, the matter embodied in this dissertation has not been submitted to any other university/ institute for award of any Degree or Diploma.

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ABSTRACT

In the contemporary scenario, where the global community is perplexed with burgeoning oil prices, depleting fossil fuel reserves and global warming, *Jatropha curcas* can prove to be a panacea to humanity. Due to its enormous potentials of substituting oil fossil fuels and saving ecological system from further deterioration, its production and propagation has paramount importance. Conventional breeding methods are not adequate enough for this. Only the application of plant tissue culture and transformation techniques can expedite this process. The prerequisite for this is the establishment of efficient regeneration protocols. Therefore, through the present study our focus was to establish *Jatropha curcas* germplasm under *in vitro* conditions. Various media formulations were used to establish the *Jatropha curcas* germplasm using the embryo excised from mature seeds. Direct regeneration of the *Jatropha curcas* was partially accomplished using various field grown explants which include leaves and internodal stem segments nurtured on MS media fortified with various combinations of auxins and cytokinins. Also the indirect organogenesis was initiated through the callus generated from the leaf explants. Studies were also made on the sensitivity of *Jatropha curcas* L. for various antibiotics. For this *Jatropha curcas* embryos were nurtured on Murashige and Skoog's medium containing antibiotics. Along with the sensitivity, the growth characteristics were also recorded so as to select the markers for *Agrobacterium*-mediated genetic transformation. Endeavours were also made to establish a protocol for the *Agrobacterium*-mediated genetic transformation of *Jatropha curcas* L. using leaf explants and internodal stem segments. In the conclusion, this study gives useful insight of the protocols for the direct and indirect regeneration of the *Jatropha* harnessing various explants, the sensitivity of the *Jatropha* plant to various antibiotics and the transformation of *Jatropha* through *Agrobacterium tumefaciens*.

Keywords: *Jatropha* germplasm, Plant growth regulators, Organogenesis, Media formulations, *Agrobacterium tumefaciens*, Genetic transformation.

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ABBREVIATIONS

2,4-D	2,4-Dichlorophenoxyacetic acid
B100	100 % biodiesel
B20	20 % blend of biodiesel with fossil diesel
BA	N^6 benzyladenine
BAP	6-Benzylaminopurine
GHG	Greenhouse gases
IBA	Indole-3-butyric acid
IAA	Indole-3-acetic acid
Kn	Kinetin
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
NO_x	Nitrogen oxides
TDZ	Thidiazuron
YEM	Yeast extract mannitol

CHAPTER 1

INTRODUCTION

In today's modern era, where the world is developing at a very fast pace, our energy resources are depleting very rapidly. Today, whole world is suffering from energy crisis because we are dependent upon extinguishable sources of energy. As a result, the whole world is suffering from increase in crude oil prices, depleting reserves of petro-fuels, global warming and political unrest. Therefore the utilization of energy crops as a renewable source of energy can only help in solving the current ecological and economic issues at both national and global scale. Energy crops are the plants grown to make biofuels such as bioethanol, biogas, biodiesel etc. The biofuels derived from plant species are the major renewable source of energy and provides 14 % of the world's energy needs. They are the high value commercial crops that play a vital role in economy of producing countries. Based on the source, biofuels are classified into following 3 generations:

First generation biofuels: The fuel generated from edible crop plants like corn, wheat, maize, soybean, sunflower, sugarcane, *Brassica*, palm oil, rapeseed, canola oil etc (Pinto et al. 2005; Demirbas 2006; Cahoon 2003; Edem 2002; Gunstone 2004).

Second generation biofuels: The fuel generated from non-edible crop plants like *Miscanthus*, *Panicum virgatum* or switchgrass, *Calotropis gigantia* or ark, *Hevea brasiliensis* or rubber seeds, *Euphorbia tirucalli* or sher, *Jatropha curcas* or ratanjyot, *Pongamia pinnata* etc (Shay 1993; Ma and Hanna 1999; Ramadhas et al. 2004; Demirbas 2003; Azam et al. 2005; Karmee and chadha 2005; Lohia 2006; Mandal and Mithra 2006).

Third generation biofuels: The fuel produced by the microbes like *Escherichia coli*, *Saccharomyces cerevisiae* (Kalscheuer et al. 2006) and microalgae like *Botryococcus braunii*, *Chlorella sp.*, *Dunaliella tertiolecta*, *Sargassum*, *Gracilaria* etc (Becker 1994; Cristi 2007, Miao and Wu 2004; Qin 2005).

As per the literature study it is evident that the food crops comprise about 30-40 % of the biofuel production worldwide, which causes county's food price inflation economy. Instead if we use second generation biofuels it can reduce these food inflations. Moreover they require less agronomic inputs like fertilizers, plowing and pesticides and their environmental impact is very less as compared to first generation biofuels.

1.1 Biodiesel-fuel for a cleaner tomorrow

Biodiesel is a diesel extracted from biological sources like vegetable oil, animal fats, and algae etc, containing long chain alkyl-esters. These fatty-acids (triglycerides) in the vegetable oil undergo transesterification reaction with alcohol to produce biodiesel and glycerol as shown in Fig. 1. Hence we can say that biodiesel is the mono-alkyl ester of vegetable oils or animal fats.

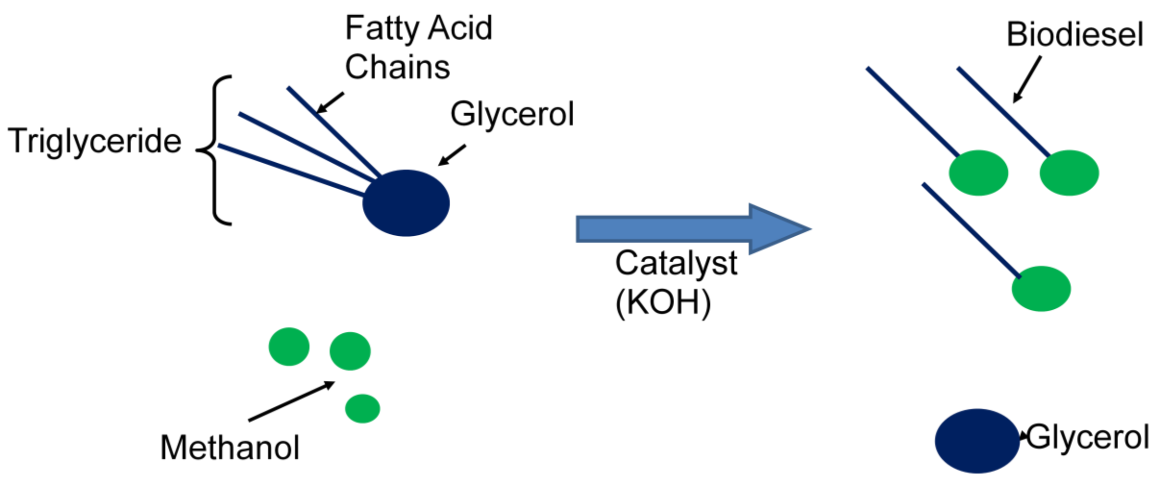


Fig. 1 Transesterification reaction to produce biodiesel

There are many products of this transesterification reaction:

a. Methyl ester (Biodiesel): 86 %
b. Glycerol: 10 %
c. Alcohol: 4 %

The glycerol produced is used for soap production or can be refined and sold as glycerin, which can be used in immense range of products including toothpaste, cosmetics, embalming fluids and pipe joint cement etc (Berchmans and Hirata, 2008). The alcohol produced can be reused for the transesterification reaction.

1.2 Properties of biodiesel

Biodiesel is a renewable source of energy, because of its plant origin. It is non aromatic and contain negligible amount of sulphur (<0.001 %). The cetane number of biodiesel is higher than that of conventional diesel. Biodiesel doesnot emit Sulphur/CO on burning, therefore, it is a non-polluting, biodegradable and environmentally safe. It is considered “carbon neutral” because the carbon dioxide emitted is nearly equal to the carbon dioxide used for the growth of plant (Baranwal and Sharma, 2005). It has lower mutagenic potency than commercial diesel (Bunger et al. 2001). It decreases green house gas emissions upto 50 % (Mukherjee et al. 2011).

1.3 Sources of biodiesel

There are various sources of biodiesel including vegetable oils, animal fats, cooking waste and algae. Different countries use different sources depending upon the availability (Table 1.). As per (Bajpai and Tyagi, 2006), various sources of biodiesel in different countries are:

Table 1. Sources of biodiesel in different countries

Country	Source of biodiesel
USA	Soybean
Brazil	Soybean
Europe	Rapeseed oil
Spain	Linseed and olive oil
France	Sunflower oil
Italy	Sunflower oil
Ireland	Animal fat
Indonesia	Palm oil
Malaysia	Palm oil
Germany	Rapeseed oil
China	Guang pi
South America	*Jatropha*

These are the edible oils, which can be used only if they are in a surplus. But in case of developing countries like India where there is shortage of food, these edible oils cannot be used for biodiesel production. Therefore the focus is to be shifted to non-edible crops like *Jatropha curcas.*

1.4 Limitations of biodiesel

Although biodiesel is a plant based renewable source of energy but still it competes with the food crops and fertile land for its production. There is a regular requirement for fertilizers and water for irrigation of fuel plants hence it is very costly. Moreover there is a wide difference in the fuel characteristics as compared to petro-diesel. The biodiesel tends to solidify at cold temperature conditions (4-5 °C). Its viscosity and surface tension is higher than the normal diesel which results in higher NO_x emissions.

1.5 Biodiesel in India

India is the second most populated country in the world with approximately 1.237 billion people (2012) and the fifth largest energy consumer in the world. 70 % of the crude oil is imported from around the world and by 2030 it is expected to import 90 % of the crude oil. Hence biofuels is the only potential opportunity that can reduce the country's dependence on foreign energy imports. The Indian government and the World Bank estimated that 26 % of India's population was classified as poor. Therefore the use of edible crops for biofuel production is not an option. Hence the government of India has to shift its interest to second generation biofuels (non-edible crops). *Jatropha curcas* has been found the best answer to this problem as it is a non-edible crop hence solves the food v/s fuel debate. It can be grown on marginal land and around crops as a protective barrier without competing with them for natural resources. India has approximately 41.93 million hectares of wasteland which has been identified as potential areas for *Jatropha curcas* plantation. In September 2008, the Indian government announced the National Biofuels Policy. This policy aims to blend conventional fuels with 20 % bioethanol or biodiesel by 2017. The railway line between Delhi and Mumbai has been planted with *Jatropha curcas* and the train itself runs on 15-20 % blend of biodiesel.

1.6 *Jatropha curcas*: Green gold in a shrub

Jatropha is a large genus of flowering plants comprises of more than 175 species of the plant belonging to the Euphorbiaceae. The commonly occurring species in India are *J. curcas, J.*

glandulifera, *J. gossypiifolia*, *J. multifida*, *J. nana*, *J. panduraefolia*, *J. integerrima*, *J. villosa*, *J. podagrica*, *J. tanjorensis*. Most of them are natives of South America and Central America. Among them *Jatropha curcas* is widely cultivated as energy crop for biodiesel production. Because of its hardiness, ease of conventional propagation and drought endurance it has spread beyond its original distribution. It is commonly named as ratanjyot, jamalgota, danti, safed arind, physic nut etc.

Table 2. Taxonomic hierarchy of *Jatropha curcas*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Malpighiales
Family	Euphorbiaceae
Subfamily	Crotonoideae
Tribe	Jatropheae
Genus	*Jatropha*
Species	*Curcas*

Fig. 2 a *Jatropha curcas* plant in fields. **b** *Jatropha curcas* plant bearing fruits

1.7 Botanical features of *Jatropha curcas*

Jatropha curcas L. is a small evergreen shrub, deciduous and diploid (2*n* = 22) of 4 to 5 meters height. In India it is found in almost all states. Gujarat, Rajasthan, Maharashtra and Tamil Nadu are the main states cultivating this crop. It had a smooth gray bark, which exudes whitish colored, watery, latex when cut. It has large green to pale-green leaves arranged alternatively with terminal inflorescence. The leaves are 6 x 15 cm, three-to five-lobed with a spiral phyllotaxis. The inflorescence is formed in the leaf axil. Flowering occurs during the wet season. In permanently humid regions, flowering occurs throughout the year. Fruits are produced in winter when the shrubs are leafless, or it may produce several crops during the year if soil moisture is good and temperatures are sufficiently high. Each inflorescence yields a bunch of approximately 10 or more ovoid fruits. The seeds are engraved in grey brown capsule of about 4 cm long, divided into 3-4 cells, each containing one seed. Seeds are black, about 2 cm long and 1 cm thick. The seeds contain about 21 % saturated fatty acids and 79 % unsaturated fatty acids. Seeds yield around 25-40 % oil by weight. The seeds also contain other chemical compounds, such as saccharose, raffinose, stachyose, glucose, fructose, galactose, and protein. The oil is largely made up of oleic and linoleic acids. *Jatropha curcas* is a toxic plant containing curcin and phorbol ester as toxins. It has a short gestation period and yields more than 2.0 kg seeds per plant per year. The tree life is of about 30 years.

1.8 Physical adaptability of *Jatropha curcas*

Jatropha curcas can be grown on marginal wastelands due to its ability to get adapted to vast agro-climatic conditions. *Jatropha* occurs mainly at the lower altitudes (0-500 m) in areas with average annual temperature above 20 °C. But it can also be grown at higher altitudes and can tolerate slight frost conditions. *Jatropha* can grow in dried regions of the tropics but grows best under a wide range of rainfall regimes from 250 to over 1200 mm per annum (Katwal and Soni 2003). Under dried conditions although the plant can grow but its photosynthesis activity is reduced. Similar is the case with very heavy rainfall. *Jatropha curcas* can grow on a wide range of soils in tropical and sub-tropical parts of the globe. It can grow on degraded soil having low fertility, on stony, gravelly and shallow and even on calcareous soils. For economic returns soil with moderate fertility is preferred.

1.9 Uses of *Jatropha curcas*

Jatropha curcas is actually a green gold in a shrub. It has numerous benefits which include:

- *Biodiesel production*: *Jatropha curcas* has relatively high seed oil content ranging from 30-40 % and lipid composition similar to that of fossil fuels. Moreover it does not compete with edible oil supplies (Jha et al. 2007; Deore and Johnson 2008). Therefore it can be best used for biodiesel production by blending it with methanol (Gubitz et al. 1999). The biodiesel so produced can be used in existing diesel engines upto 20 % blend (B20).
- *Living fence*: *Jatropha curcas* has been cultivated as a living fence around the crops against live stocks influx and grazing (Jones and Miller 1991). It is also grown on marginal lands to control soil erosion by wind or water (Heller 1996).
- *Medicinal uses*: *Jatropha* is known for its use as a purgative/laxative. All parts of plant are used in traditional and folk medicines and for veterinary purpose (Duke 1988). The methanol extract from leaves have Betablockers which have cardiovascular action in humans. The sap from stems can cure bleeding wounds. The latex has several alkaloids with anti-cancer properties (Van den Berg et al. 1995; Thomas et al. 2008). The roots act as antidote for snake-bites and have anti-inflammatory activity (Mujumdar and Misra 2004). Oil from *Jatropha curcas* seeds is used in skin ailments and in treatment of rheumatism (Heller 1996).
- *Insecticide:* All parts of the *Jatropha curcas* plant show insecticidal properties against various insects like cotton bollworm, and for pests like pulses, potato and corn (Kaushik and Kumar 2004).
- *Soap Production: Jatropha* oil is widely used by local soap industries to produce soaps. Glycerol, the by-product of transesterification reaction in synthesis of biodiesel can be used to produce high quality soap. The soap has positive effects on skin and is therefore marketed for medicinal purposes (Berchman and Hirata 2008).
- *Use of Jatropha curcas seed cake:* The seed cake containing curcin cannot be used as a feedstock for animals but can be used as potential organic manure since it has nitrogen content ranging from 3.2-3.8 % (Kumar and Sharma 2008).

1.9 Propagation of *Jatropha curcas* L.

There are mainly three modes of propagating *Jatropha curcas* plant. First is sexual propagation through seeds. Here high quality seeds are selected, usually heavy and large seeds, and planted in soil at about 2 cm depth. The seeds are supplied with high humidity, air, fertilizers etc. It usually results in good quality plants with optimum root development. The second mode of propagation is through cutting. It is an asexual propagation where thick branches of about 30 cm length are placed directly in wet fertile soil. These branches regenerate into a new well developed plant. Hence we can have a clone of a single plant. It is the cheapest source of propagation. But these plants have only lateral roots therefore they cannot access nutrients in deep soil. Third method of propagation is micropropagation. It is a technical method to produce large amount of genetically identical plants using various plant growth hormones in the laboratory. It usually results in good quality plants but this method is very costly. Here we can grow plant *in vitro* conditions and can genetically modify it.

1.10 Biotechnological aspects of *Jatropha curcas*

There are many problems associated with *Jatropha curcas* plant. The oil yield is very low. Although the plant can grow on marginal lands but such plants only produces marginal amount of oil. For proper oil production they need proper water and irrigation. The plant is very toxic containing toxins like curcin and phorbol ester, which limits its applications in many areas like as cooking oil, as a feedstock for animals etc. Therefore focus is made now a days to improve its traits like making it non-toxic, increasing the oil yield, making it resistant to pests and adaptable to different agro-climatic conditions. Therefore the plants are micropropagated in laboratory conditions and their genetic transformation studies are being carried out.

1.11 *Agrobacterium*-mediated Genetic transformation

Agrobacterium tumefaciens is a rod shaped gram negative soil bacterium which infects the plants and causes crown gall disease. It contains a plasmid called Ti plasmid which contains virulent genes in its T-DNA (Fig. 3). The wounded part of the plant produces acetosyringone that activates the virulence genes which causes the T-DNA part to be transferred to plant cell, where it gets incorporated at random location in the plant genome.

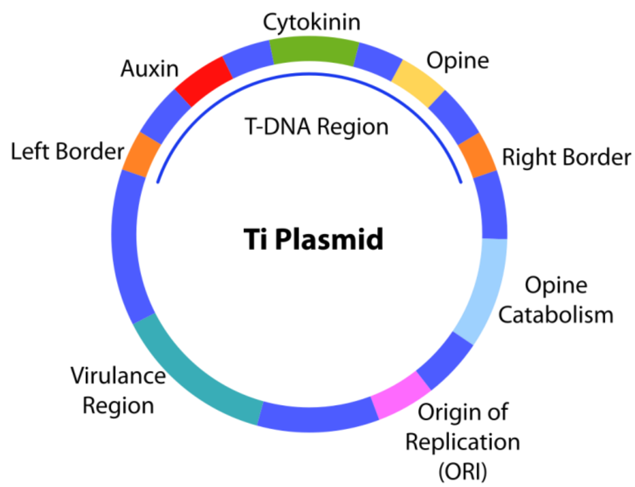


Fig. 3 Schematic diagram of Ti-plasmid present in *Agrobacterium tumefaciens*. (Source: Wikipedia)

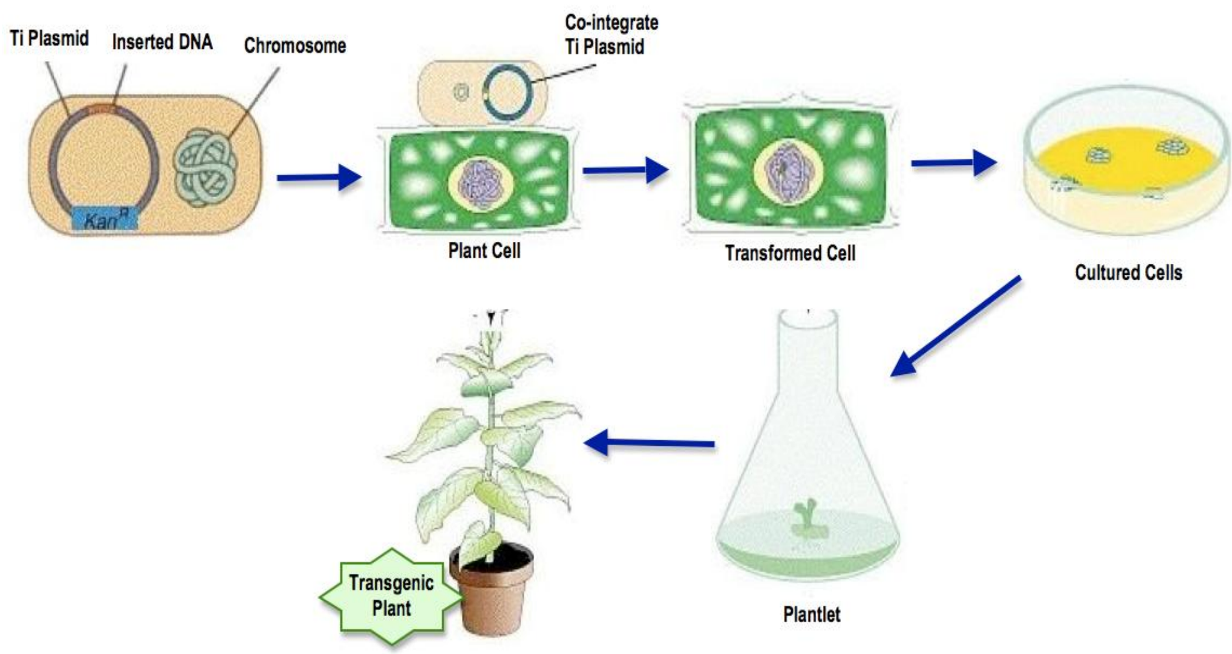


Fig. 4 Schematic diagram of *Agrobacterium*-mediated genetic transformation (Source: Wikipedia)

First step in *Agrobacterium*-mediated gene transfer is to prepare competent cells containing gene of interest. For this the gene of interest is inserted into the T-DNA of Ti plasmid along with the gene of interest a selection marker gene (say Kanamycin resistant gene) is also incorporated into the T-DNA. Then the explants like leaf-discs or internodal stem segments are co-cultivated with the above made competent cells for about 2 to 3 days. The wounded area of the leaf-disc will produce acetosyringone which will activate the transfer of T-DNA to the host genome. The transformed cells are selected using the selection markers and are regenerated into a new plant.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Conventional propagation of *J. curcas*

Conventionally *Jatropha curcas* is propagated by 2 means, by seeds and by cuttings. *J. curcas* is a spermatophyte i.e. a seed bearing plant which can produce about 1-2 kg seeds per plant per year at an age of 2-3 years. The production amount may increase with the age of the plant. But the yield per year is uncertain and it may not produce optimal yield for several years, making it unsustainable. Since the plant is cross-pollinated therefore the seeds are of unknown genetic potential (Mukherjee et al. 2011). The trees are also propagated by stem cuttings. But the trees so produced showed lower longevity and possessed a lower drought and disease resistance than those propagated by seeds. The trees produced from cuttings often produced pseudo-taproots (Sujatha et al. 2005). Problems associated with seed cultivation (Mukherjee et al. 2011)

- Heterozygosity
- No genetic uniformity in planting material
- Seasonal practice and the seed-borne diseases may be transmitted to the seedling.
- Cannot ensure the quality in terms of oil content as the plant is heterozygous.

Thus propagation through seeds is not reliable and vegetative propagation by stem cutting is inadequate to meet the demand. This indicates the need for developing micropropagation methods so as to facilitate the large scale production of true-to-type elite plants.

2.2 *In vitro* plant regeneration of *Jatropha curcas*

Regeneration is the key tool of plant biotechnology. It works on the principle based on the totipotent nature of plant cells (Haberlandt 1902) i.e. the ability of single cell to grow into an entirely new plant. In *in vitro* plant regeneration, the plant cells regenerate in a medium containing the required nutrients and growth regulators (PGRs). Almost all types of explants are now days used for regeneration which include embryo, shoot tip (Rajore and Batra 2005), nodal segments (Sujatha et al. 2005), leaf explants (Sujatha and Mukta 1996; Sujatha et al. 2005, Khurana-Kaul et al. 2010; Reddy et al. 2008; Divakara et al. 2010), petiole (Kumar and Reddy 2010; Kumar et al. 2011), hypocotyls (Sujatha and Mukta 1996) , epicotyls (Kaewpoo and Te-chato 2010) and cotyledons

(Kumar et al. 2010). Murashige and skoog's (MS) medium was the foremost preferred medium to initiate and improve the response in *in vitro* cultures. Various growth regulators are added to this MS medium to enable plant regenerate. *In vitro* plant can be regenerated in 2 ways: Direct regeneration without any intervening callus phase and indirect regeneration with intervening callus phase. This technique can also be used for the genetic improvement of the plant.

2.3 Micropropagation using nodal segments

Nodal meristems are an important source tissue for micropropagation. The plants raised from these are more resistant to genetic variations (Pierik 1991). Nodal segments are most frequently used for direct induction of shoot buds. Various studies have been made on to optimize the regeneration protocol using nodal segments as explants.

Kalimuthu et al. (2007) reported the best shoot bud induction in nodal explants when cultured on MS basal medium fortified with 22.19 µM BAP, 2.32 µM Kn and 0.57 µM IAA. According to Shrivastava and Banerjee (2008), shoot regeneration from axillary nodes was observed on MS medium containing 13.31 µM BAP, 4.90 µM IBA along with growth additives adenine sulphate, glutamine and L-arginine. They also reported the role of auxins in multiple shoot induction. The higher concentration of auxins was found inhibitory to morphogenesis. Hence the use of appropriate auxin-cytokinin ratio is essential for proper shoot and root formation. Datta et al. (2007) found that MS medium supplemented with 22.19 µM BAP and 108.58 µM adenine sulphate was more suitable for regeneration from nodal explants.

The effect of orientation of the explants on the shoot bud formation was also reported by Kumar et al. (2011). They reported that the number of shoot buds induced per explants were higher when the explants was cultured in the horizontal position.

2.4 Direct regeneration using leaf explants

Cotyledonary leaf tissues are the most responding explants in *J. curcas*. They have greater multiplication potential as compare to nodal segments and shoot tips. Various studies have been reported for direct regeneration of *J. curcas* plant using leaf explants. Sujatha and Makkar (2005) have reported direct adventitious shoot bud regeneration from leaf segments using a combination of 22.19 µM BAP and 4.90 µM IBA in MS medium.

Deore and Johnson (2008) first reported the role of TDZ in inducing high frequency shoot buds formation in leaf disc cultures without any intervening callus phase. Maximum shoot bud formation was observed on MS medium fortified with 2.22 µM BAP, 2.27 µM TDZ and 0.49 µM IBA. When BAP alone was used in the absence of TDZ only callus formation occurred. This signifies the role of TDZ in short bud formation. The role of TDZ in direct regeneration was also reported by Kumar and Reddy (2010), Kumar et al. (2011) and Khemkladngoen et al. (2011). Khurana-Kaul et al. (2010) reported the effect of high copper content along with 0.98 µM TDZ and 0.98 µM IBA on direct shoot bud regeneration from leaf explants. According to this high copper content enhances the shoot bud induction by 10 times.

Kumar et al. (2010) reported direct shoot bud formation on cotyledonary leaf explants using MS medium supplemented with different concentrations of TDZ and other growth regulators. The elongated shoots 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.

Zhang et al. (2013) also reported direct shoot bud from mature leaves on MS medium supplemented with 1.0 mg/l TDZ, 0.5 mg/l Kn and 0.5 mg/l GA_3. The induced shoots were elongated on MS medium with 0.3 mg/l BAP and 0.01 mg/l IBA. The roots were established in half-strength MS medium containing 2 mg/l IBA. The shoot bud induction rate with this method was reported to increase from 53.5 % to 90 %.

2.5 Micropropagation of *Jatropha curcas* using shoot tips as explants

Rajore and Batra (2005) have reported direct regeneration of shoot buds from shoot tips of *J. curcas* on MS medium supplemented with 8.87 µM BAP and 2.85 µM IAA along with adenine sulphate, glutamine and activated charcoal. The regenerated shoots were rooted on half-MS containing 2.46-24.6 µM IBA. Highest frequency of rooting was observed on MS medium supplemented with 4.7 µM IBA. With this protocol almost 60-70 % of the acclimatized plants survived.

2.6 Indirect organogenesis

Callus induction can be achieved from various parts of the plant which include leaf, stem, node, petiole, cotyledon and hypocotyls (Kumar and Sharma 2008). Callus can be induced from leaf explants at higher concentrations of BAP (5.0 mg/l) with NAA (1.0 mg/l) within 3-4 weeks (Rajore and Batra 2007). NAA (1.0 – 4.0 mg/l) alone in half MS medium can also induce callus. This finding suggests that *Jatropha curcas* is highly sensitive to auxins with respect to induction of cell division rather than induction of roots (Shrivastava and Banerjee 2008). Replacing NAA by IBA can also induce callus (Deore and Johnson 2008). Varshney and Johnson (2010) reported that the immature embryo cultured on different combinations of auxins and cytokinins showed a good response to callus induction and subsequent plant regeneration.

2.7 *Agrobacterium*-mediated genetic transformation of *J. curcas*

Genetic transformation for improving the traits is a valuable method for developing new *J. curcas* varieties. Using this technique various improvements can be made in *J. curcas* plant such as the oil biosynthesis pathway can be improved; the plant can be made non-toxic and resistant to biotic (bacterial, fungal and viral disease) and abiotic stresses (temperature, humidity, salinity, humidity). Wide research is going on to establish standard protocol for the genetic transformation of *Jatropha curcas* plant. The development of an efficient regeneration system amenable to genetic transformation is a prerequisite for genetic engineering. Research for the establishment of standardized regeneration protocol is still going on.

Li et al. (2008) has established an efficient genetic transformation procedure from the Chinese accessions of *Jatropha curcas* for the first time via *Agrobacterium tumefaciens* infection of cotyledon disc explants. As a result about 55 % of the cotyledon explants produced phosphinothricin-resistant callus on MS medium containing 1.5 mg/l BA, 0.05 mg/l IBA, 1 mg/l phosphinothricin and 500 mg/l cefotaxime after about 4 weeks. The callus was then shifted to regeneration medium containing 1.5 mg/l BA, 0.05 mg/l IBA, 0.5 mg/l GA_3, 1 mg/l phosphinothricin and 250 mg/l cefotaxime. About 33 % of the resistant calli could differential into shoots.

Kumar et al. (2010) has reported a protocol for the stable genetic transformation of *Jatropha curcas* via *Agrobacterium tumefaciens*-mediated gene transfer using the leaf explants. They found that various parameters such as preculture of explants, wounding of leaf explants, *Agrobacterium* growth phase, infection duration, co-cultivation period, co-cultivation medium pH and acetosyringone etc effect the efficient transformation. Using *Agrobacterium* strain LBA 4404 containing binary vector pCAMBIA 1304, hygromycin-phosphotransferase (hpt) gene was transferred. Maximum transformation was achieved using 4-day precultured, non-wounded leaf explants infected with *Agrobacterium* culture corresponding to OD_{600} = 0.6 for 20 minutes, followed by co-cultivation for 4 days in a co-cultivation medium containing 100 µM acetosyringone, pH 5.7. Co-cultivated leaves were then cultured on MS medium supplemented with 2.27 µM TDZ and 4.5 µM hygromycin.

Subroto et al. (2014) also standardized the protocol for regeneration and *Agrobacterium*-mediated genetic transformation of *Jatropha curcas* using cotyledon explants. They used *A. tumefaciems* strain LBA4404 containing binary vector pCAMBIA 1303, having a reporter gene called *gus* gene. The fresh cotyledons were extracted from embryos and soaked with *A. tumefaciens* for 20 minutes followed by co-cultivation on MS medium for three days. The cotyledons were then decontaminated with 100 mg/l timentin and 0.2 % plant preservation mixture. The explants were then transferred to callusing media containing 1.5 mg/l BAP and 0.1 mg/l IBA and then transferred to shoot induction medium containing 2 mg/l BAP, 0.05 mg/l IBA and 0.5 mg/l GA_3. This resulted in 45 % of the shoot induction. The transformants were analyzed with *GUS* assay.

CHAPTER 3

AIM OF THE PRESENT STUDY

3.1 Aim of the present study

Literature survey clearly indicates that the full potential of *Jatropha curcas* as a biodiesel crop is not fully realized yet. One of the major reasons is the lack of availability of genotypically/genetically improved *Jatropha* germplasm particularly with regard to crop yield and oil content of the seeds. Moreover for cultivation of *Jatropha* we cannot afford prime agricultural land. Therefore there is a growing need of the *Jatropha* varieties that can grow in the semi-arable marginal lands, degraded soils and also can withstand drought, flood and other challenging biotic and abiotic stresses. Therefore, quality and quantity improvement of this crop through transgenics is a need of the hour. Again the success of various genetic transformation protocols is based on *in vitro* tissue culture aspects of this commercially important crop. In other words, we require both standard regeneration and transformation protocols. Therefore the aim of the study was to standardize a suitable and easy-to-execute protocol for the establishment of *Jatropha* germplasm under *in vitro* conditions, along with regeneration from the suitable explants namely shoot tips, nodal explants and leaves collected from the elite field-grown plants. Moreover, efforts were made for the *Agrobacterium*-mediated genetic transformation of *Jatropha curcas*. In the light of the above points, following objectives were framed.

3.2 Objectives

- *In vitro* establishment of *Jatropha curcas* L. germplasm using embryo culture techniques
- Direct regeneration of *Jatropha curcas* L. using field grown explants like leafs, shoot tips, nodes and internodes
- Indirect organogenesis or callus mediated shoot bud regeneration of *Jatropha curcas* L.
- Sensitivity tests of *Jatropha curcas* L. for various antibiotics
- To establish the protocol for *Agrobacterium*-mediated genetic transformation of *Jatropha curcas* L.

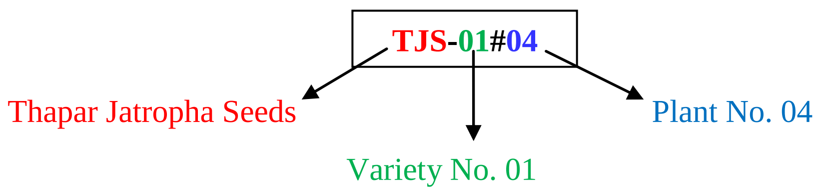
CHAPTER 4

MATERIALS & METHODS

4.1 Materials

4.1.1 Procurement of *Jatropha curcas* L. plant material

The plant material and the explants required for the tissue culture work were collected from the elite *Jatropha curcas* L. germplasm bank maintained at Thapar University, Patiala. For seeds collection the best performing *Jatropha curcas* L. genotypes in the field conditions on the basis of seed yield and seed oil content were selected i.e TJS-01#04, TJS-23#17. Shoot tips and nodal segments were also collected from TJS-01# 04 and TJS-23#17.



4.1.2 Chemicals and Plant growth regulators (PGRs)

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

4.1.4 Glass wares and plastic wares

Various glass wares and plastic wares were used, which include 100 ml, 150 ml, 250 ml, 500 ml and 1000 ml conical flasks, measuring cylinders 25 ml, 100 ml and 500 ml., beakers 250 ml and 500 ml, culture bottles (8 x 3 inches) with plastic caps. All these were brought from Borosil Products Pvt. Ltd. Appendorfs (1.5 ml, 2 ml), tips (200 µl and 1000 µl) were brought from Tarsons Products Pvt. Ltd.

4.1.5 Other Materials

Glass plate, scalpel, forceps, blades, tissue paper, Cling film, ethanol, detergent, cotton etc

4.1.6 Bacterial strains and plasmids

- *Agrobacterium tumefaciens* (LBA4404) strain: LBA4404 (Ach5 pTiAch5) Sm/Sp(R) in the virulence plasmid (from Tn904); all T-DNA of pTiAch5 eliminated in pAL4404. LBA4404 strain was maintained on YEM medium containing rifampicin (15 µg mL^{-1}) and streptomycin (50 µg mL^{-1}).
- Plasmid: A binary plasmid pBI121 containing kanamycin resistant gene.

4.1.7 Media preparations

a) Murashige and Skoog (MS) media

It is a plant growth media, invented by plant scientists Toshio Murashige and Folke K. Skoog in 1962. It is selected as the optimum plant culture media. It has very high concentration of nitrate, potassium and ammonia salts. The following table shows composition of MS basal medium (Table 3).

Table 3. Composition and Stock preparation for MS Basal Medium

I	**Major Salts**			
S.No.	**Components**	**MS Basal Conc. (mg/l)**	**Amount Required 100X Stock (g/l)**	**For 1L medium (ml)**
1	KNO_3	1900	190	10
2	NH_4NO_3	1650	165	10
3	$MgSO_4.7H_2O$	370	37	10
4	$CaCl_2.2H_2O$	440	44	10
5	KH_2PO_4	170	17	10
II	**Minor Salts**			
1	H_3BO_4	6.2	0.62	1
2	$MnSO_4.4H_2O$	22.3	2.23	1
3	$ZnSO_4.7H_2O$	8.6	0.86	1
4	$Na_2MoO_4.2H_2O$	0.25	0.025	1
5	$CuSO_4.5H_2O$	0.025	0.0025	1
6	$CoCl_2.6H_2O$	0.025	0.0025	1

7	KI	0.83	0.083	1
8	$Fe_2EDTA.2H_2O$	30	3	1
III	**Vitamins**			
1	Nicotinic acid	0.5	0.5	1
2	Pyridoxine HCl	0.5	0.5	1
3	Thiamine HCl	0.1	0.1	1
4	Glycine	2.0	2.0	1
5	Myo-inositol	100	100.0	1

b) YEM media

Table 4. Composition and stock preparations for YEM medium

S.No.	Components	Amount per liter (g)	Amount for 100 ml medium (mg)
1	Yeast extract	0.4	40
2	Mannitol	10	1000
3	$MgSO_4.7H_2O$	0.2	20
4	K_2HPO_4	0.5	50
5	NaCl	0.1	10

For preparation of YEM-kanamycin medium 50 mg/l of kanamycin was added to the YEM medium. For making YEM agar medium 1.5 % agar is added to the medium.

4.2 Methods

4.2.1 Preparation of MS medium

The tissue culture bottles were washed and kept overnight in hot air oven at 70 °C. For preparing 1L MS media, took 1L flask and added ~300-400 ml of double distilled water. Then all the major salts, minor Salts and vitamins were added in a proportion given in Table 3. Sucrose 2.5 % i.e. 25 g in 1L was added and mixed well. The volume was made-up to 1L with double distilled water and the pH was adjusted to 5.7 using freshly prepared 1N NaOH. Agar 0.7 % i.e. 7 g in 1L medium was added to the media for solidifying it. The media was then heated in microwave for approximately 10 minutes, with continuous shaking after every 1 minute, till the agar melted completely and the media started to boil. Approximately 30-35 ml of the media was then poured in each tissue culture bottle of 8 x 3 inches. The bottles were capped tightly and were autoclaved at temperature 121 °C, pressure 15 psi for 15 minutes. After autoclaving the media was left to cool and solidify. Then the bottles containing media were shifted to plant growth room for 2 days so as check for any bacterial or fungal contamination.

This MS medium either alone or in combination with various plant growth regulators (cytokinins and auxins) was used for carrying out the tissue culture work. Various media formulations were done so as to achieve the successful regeneration.

Some of the common media formulations used in this project are:

Table 5. Media formulations for Antibiotic sensitivity tests of *J. curcas*

Media	Composition
M1	MS Basal Medium (MS)
M2	MS + 50 mg/l Amikacine
M3	MS + 100 mg/l Amikacine
M4	MS + 50 mg/l Amoxicillin
M5	MS + 100 mg/l Amoxicillin
M6	MS + 50 mg/l Ampicillin
M7	MS+ 100 mg/l Ampicillin
M8	MS + 50 mg/l Cefoperazone

M9	MS + 100 mg/l Cefoperazone
M10	MS + 50 mg/l Cefotaxime
M11	MS + 100 mg/l Cefotaxime
M12	MS + 50 mg/l Ceftriaxone
M13	MS + 100 mg/l Ceftriaxone
M14	MS + 50 mg/l Chloramphenicol
M15	MS + 100 mg/l Chloramphenicol
M16	MS + 50 mg/l Ciprofloxacin
M17	MS + 100 mg/l Ciprofloxacin
M18	MS + 50 mg/l Gentamicin
M19	MS + 100 mg/l Gentamicin
M20	MS + 50 mg/l Hydrocortisone Sod. Succinate
M21	MS + 100 mg/l Hydrocortisone Sod. Succinate
M22	MS + 50 mg/l Kanamycin
M23	MS + 100 mg/l Kanamycin
M24	MS + 50 mg/l Ofloxacin
M25	MS + 100 mg/l Ofloxacin
M26	MS + 50 mg/l Pantoparazole
M27	MS + 100 mg/l Pantoparazole
M28	MS + 50 mg/l Rifampicin
M29	MS + 100 mg/l Rifampicin
M30	MS + 50 mg/l Streptomycin
M31	MS + 100 mg/l Streptomycin
M32	MS + 50 mg/l Tetracyclin
M33	MS + 100 mg/l Tetracyclin

Table 6. Media formulations for *Jatropha curcas* regeneration

Media	**Composition**
M34	MS + 0.1 mg/l BAP + 0.01 mg/l NAA
M35	MS + 0.5 mg/l BAP + 0.01 mg/l NAA
M36	MS + 1.0 mg/l BAP + 0.01 mg/l NAA
M37	MS + 0.1 mg/l BAP + 0.1 mg/l NAA
M38	MS + 0.5 mg/l BAP + 0.1 mg/l NAA
M39	MS + 1.0 mg/l BAP + 0.1 mg/l NAA
M40	MS + 0.1 mg/l BAP + 0.1 mg/l 2,4-D
M41	MS + 0.5 mg/l BAP + 0.1 mg/l 2,4-D
M42	MS + 0.1 mg/l BAP + 0.5 mg/l 2,4-D
M43	MS + 0.5 mg/l BAP + 0.5 mg/l 2,4-D
M44	MS + 0.1 mg/l TDZ
M45	MS + 0.3 mg/l TDZ
M46	MS + 0.5 mg/l TDZ
M47	MS + 0.5 mg/l BAP + 0.5 mg/l TDZ + 0.1 mg/l IBA
M48	MS + 0.5 mg/l BAP
M49	MS + 0.5 mg/l BAP + 0.1 mg/l IBA
M50	MS + 0.5 mg/l BAP + 0.5 mg/l IBA
M51	MS + 1.0 mg/l BAP + 0.5 mg/l IBA
M52	MS + 2.0 mg/l BAP + 0.5 mg/l IBA
M53	MS + 3.0 mg/l BAP + 0.5 mg/l IBA
M54	MS + 4.0 mg/l BAP + 0.5 mg/l IBA
M55	MS + 5.0 mg/l BAP + 0.5 mg/l IBA

4.2.2 Maintenance of Aseptic conditions

Aseptic conditions were maintained throughout in the laboratory. The entire tissue culture work was carried out under strict aseptic conditions in the laminar air flow hood fitted with UV-C germicidal lamp (15 W, peak emission 2637 Å) and HEPA filters of pore size 0.2 µm. Before use the laminar air flow hood was fumigated with the formaldehyde solution. The floor of the hood was thoroughly cleaned with alcohol. Then the U.V was switched on for about 15-20 minutes. All the accessories like scalpel, forceps, glass plate, blade, gas burner etc to be used inside must be autoclaved and surface sterilized with alcohol. The hands were properly wiped with absolute alcohol before working.

4.2.3 Sterilization protocol for *Jatropha curcas* seed kernels

Mature *Jatropha curcas* L. seeds were decorticated and the kernels recovered were kept in a separate bottle. Only healthy intact and white kernels were used. The kernels were then imbibed in fresh water for about 2-3 hours so as to remove all the adherent dust particles. After imbibitions the neck of the bottle containing kernels was covered with net. Then the kernels were given the detergent treatment (Tween-20) for about 7-10 minutes with random shakings. The bottle was then placed under the running water for about 10 minutes to completely remove the detergent. The seeds were then treated with 5 % solution of sodium hypochlorite (Bactericide) for about 5-7 minutes to kill all the bacterial contaminants. The seeds were again washed with running water for about 10 minutes to completely remove the Hypochlorite. The kernels were then treated with 0.2 % bavistin (Fungicide) for about 5-7 minutes to kill all the fungal contaminants. The seeds were again washed under running tap water for about 15 minutes. Then under the laminar air flow hood the kernels were given 0.2 % mercuric chloride ($HgCl_2$) treatment for 5-7 minutes, followed by 5-6 washings with autoclaved double distilled water.

4.2.4 Embryo culturing of *Jatropha curcas* L.

Firstly the laminar air flow hood was sterilized properly as mentioned in 4.2.2. A tube with cotton at base containing absolute alcohol was placed inside the laminar hood. The glass plate, forceps, scalpel and blade were heat sterilized. Then each seed kernel was carefully placed on the sterile plate in the horizontal position. Holding the kernel with forceps the outer very thin and transparent coating on the kernel was carefully removed. Then a very fine vertical cut less than half of the seed thickness was made at almost midpoint of seed because our embryo lies horizontally exactly in the centre of the seed. Healthy and intact embryos were then isolated. These embryos were them carefully picked up using the forceps and placed on the media in a vertical position with the cotyledons facing upward. The bottles were then capped and sealed properly using a cling film. Proper labelling was made on the bottles and they were kept in the growth room under maintained conditions of temperature 23 °C and light regime of 16 hours followed by 8 hours of dark period.

4.2.6 Sterilization protocol for shoot tips, nodal segments and leaf explants

Fresh green nodal segments and shoot tips and young leafs were brought from the 6 years old *Jatropha curcas* L. plant growing in the fields of Thapar University. The explants were firstly imbibed in fresh water for about 2-3 hours so as to remove all the adherent dust particles. After imbibitions the neck of the bottle containing explants was covered with net. Then the explants were given the detergent treatment (Tween-20) for about 7-10 minutes with random shakings. The bottle was then placed under the running water for about 10 minutes to completely remove the detergent. The explants were then treated with 5 % solution of sodium hypochlorite (Bacteriocide) for about 5-7 minutes to kill all the bacterial contaminants followed by washing under running tap water for 10 minutes. The explants were then treated with 0.2 % bavistin (Fungicide) for about 5-7 minutes to kill all the fungal contaminants. The explants were again washed under running tap water for about 15 minutes. Then under the laminar air flow hood the explants were given 0.2 % mercuric chloride ($HgCl_2$) treatment for 5-7 minutes, followed by 5-6 washings with autoclaved double distilled water.

4.2.7 Inoculation of shoot tips and nodal explants

Firstly the laminar air flow hood was sterilized properly as mentioned in 4.2.2. The glass plate, forceps, scalpel and blade were heat sterilized. The shoot tip/nodal segment were carefully placed on the sterile glass plate in the horizontal position. All the pre existing leafs on the shoot tip/nodal segment were removed. The shoot tip/ nodal segment were given fresh cuts on both the ends. Dead portions were removed carefully. Then the shoot tip/ nodal segment were placed vertically on the suitable media. The bottles were capped and sealed properly with cling film. Proper labelling was made on the bottles and they were kept in the growth room under maintained conditions of temperature 23 °C and light regime of 16 hours followed by 8 hours of dark period.

4.2.8 *Agrobacterium tumefaciens*-mediated co-cultivation

For the co-cultivation *Agrobacterium tumefaciens* transformed strain LBA4404 containing plasmid p(BI121) was used. The *Agrobacterium* transformants were firstly grown on YEM-kanamycin agar plates, incubated at 28 °C for 24 hours. Single colony was inoculated in YEM-kanamycin broth and incubated for 24 hours. The culture was expected to have O.D ~ 1.0. But for co-cultivation, we require O.D < 1.0. Therefore 1 ml of culture was diluted with 5 ml of MS basal medium. Fresh explants (Leafs and internodes) of *Jatropha curcas* accession TJS-01#04 were brought from the fields of Thapar University, Patiala. Fresh cuts were made on these explants and were co-cultivated with the diluted *Agrobacterium* transformant in MS basal medium for about 20 minutes. After the co-cultivation the explants were blotted on sterile autoclaved filter paper to remove the excess bacteria. The internodal stem segments were then placed both vertically and horizontally on the MS basal medium, whereas the leaves were placed such that the abaxial side touch the media. The cultures were then incubated in dark for 48 hours in the plant growth room. After 48 hours the explants were removed from the MS medium and were washed in cefotaxime (250 mg/l) for 5 minutes so as to remove the *Agrobacterium* from the surface of the plant. The explants were then shifted to the shoot regeneration medium containing 0.5 mg/l BAP, 0.5 mg/l TDZ, 0.1 mg/l IBA, 80 mg/l kanamycin and 250 mg/l cefotaxime. The bottles were sealed and kept in plant growth room.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 Antibiotic sensitivity tests of *Jatropha curcas* L. using embryo culture techniques

The sensitivity test for various antibiotics was carried out on an embryo culture raised plantlets for the variety of *Jatropha curcas* accessions (TJS-01#04, TJS-23#17) seeds. Firstly, the black seed coat was removed from the seeds to get white seed kernels. These white seed kernels were then surface sterilized by the protocol 4.2.3. Then under the laminar air flow hood, the embryos were very carefully separated from the seed kernels using scalpel and forceps.

Fig. 5 *Jatropha curcas* **a** seeds with seed coat. **b** kernels **c** embryos

The *Jatropha curcas* embryos were then cultured on MS basal media containing various antibiotics at 2 concentrations 50 mg/l and 100 mg/l and their response were recorded after approximately one month (Table 7). The embryo culture raised plantlet on MS basal media without antibiotic was kept as a positive control. The control showed positive result and developed into a plant with proper roots, shoots and leafs. The embryo culture raised plantlet bearing antibiotic resistance grew well into a plant whereas the embryo culture raised plantlet bearing sensitivity for the antibiotic turned pale and died. The following observations were recorded for different antibiotics.

'+' means positive growth

'++' means enhanced growth and

'--' means no growth and

'-' means poor growth

Table 7. Effect of various antibiotics on the embryo raised plants of *Jatropha curcas* L.

S.No.	Antibiotic	Conc: 50 mg/l	Conc: 100 mg/l
1	Amikacine	--	--
2	Amoxicillin	+	+
3	Ampicillin	+	+
4	Cefoperazone	+	+
5	Ceftriaxone	-	-
6	Cefotaxime	+	+
7	Chloramphenicol	+	-
8	Ciprofloxacin	--	--
9	Gentamicin	--	--
10	Hydrocortisone Sod. Succinate	+	+
11	Kanamycin	--	--
12	Ofloxacin	--	--
13	Pantoparazole	-	-
14	Rifampicin	+	+
15	Streptomycin	--	--
16	Tetracyclin	++	++

The embryo cultures grew well on amoxicillin, ampicillin, cefoperazone, cefotaxime, chloramphenicol, hydrocortisone sodium succinate, rifampicin and tetracyclin. Proper development of roots, shoots and leafs was observed.

Enhanced growth as compare to control was observed in case of plant raised on MS + 50mg/l Tetracyclin and MS + 100mg/l Tetracyclin as shown in Fig. 6.

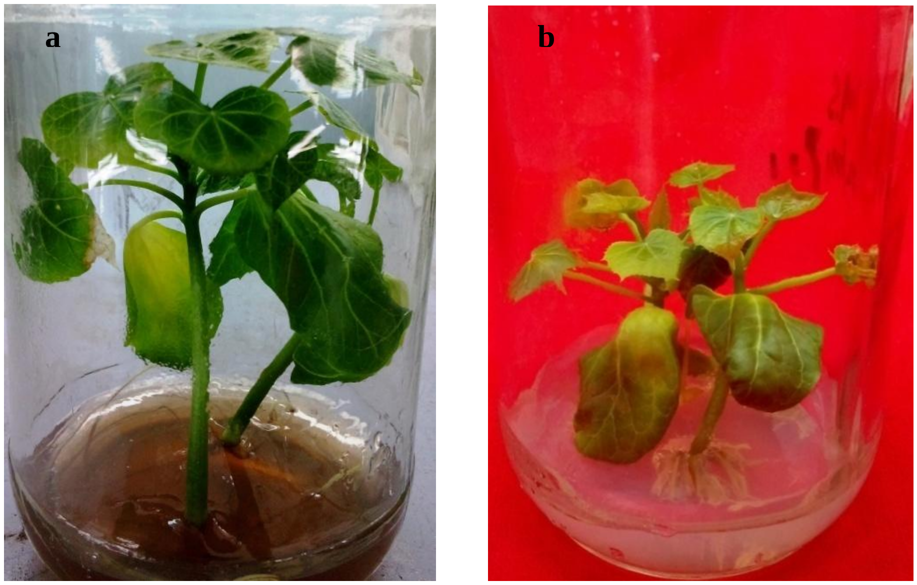


Fig. 6 Effect of tetracyclin on the embryo raised plant **a** Embryo raised plant on MS + 100 mg/l Tetracyclin. **b** Embryo raised plant on normal MS basal medium

The embryo cultures didn't grew well on amikacine, ciprofloxacin, ceftriaxone, gentamicin, ofloxacin, kanamycin, pantoparazole, and streptomycin. In case of amikacine, ceftriaxone, ciprofloxacin, gentamicin, ofloxacin, kanamycin and streptomycin, the embryo didn't responded, turned pale and finally died as shown in Fig. 7.

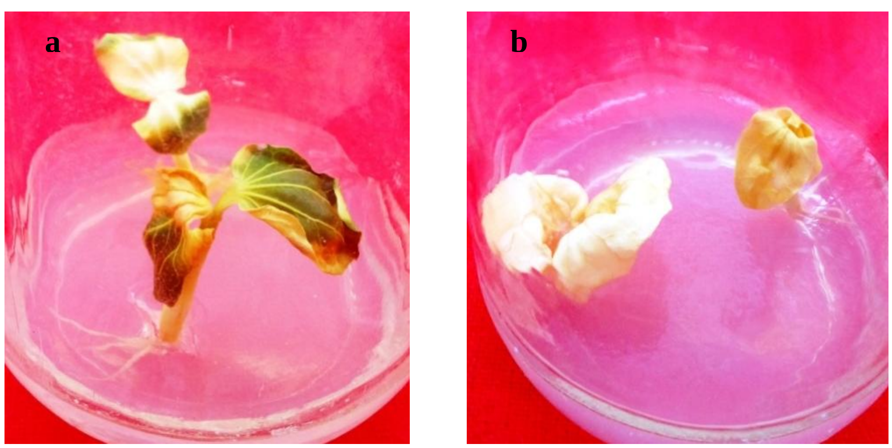


Fig. 7 *Jatropha curcas* embryos showing sensitivity to antibiotics. **a** Embryo raised plants on MS basal medium containing Streptomycin. **b** Embryo raised plant on MS basal medium containing Kanamycin.

Whereas in case of pantoparazole, the embryo grew well into a plant with proper shoots and leafs, but very poor root formation was observed. Each embryo produces about 6-8 shoots but the plant size was smaller than the control (Fig. 8).

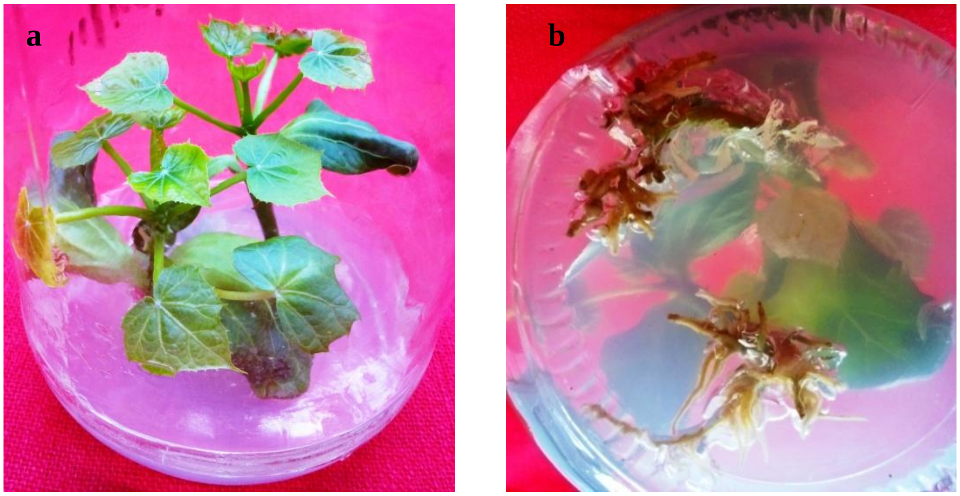


Fig. 8 Embryo raised plants on medium containing pantoparazole **a** Embryo raised plant on MS basal medium containing pantoparazole. **b** very little root formation in embryo raised plant on MS + 50 mg/l pantoparazole.

5.2 Micropropagation through Shoot tip explants

Fresh shoot tips were brought from 2 different field plants TJS-01#04 and TJS-23#17 procured from different states of Punjab. They were surface sterilized using the protocol 4.2.6. Under the laminar air flow hood, fresh cuts were made on the shoot tips so as to remove all dead ends. Different media formulations were made and were cultured with the shoot tips. Shoot tip on MS basal medium was kept as control. No regeneration was observed in control.

Shoot tips were cultured on MS medium supplemented with BAP and NAA in a concentration range of 0.1-1 mg/l. of BAP and 0.01-0.1 mg/l NAA. After 4 weeks, number of new shoots per explants was observed to increase with increase in BAP concentrations (Table 8). Maximum number of shoots was observed on MS media containing 1 mg/l BAP and 0.01 mg/l NAA and minimum number of shoots was observed on MS media containing 0.1 mg/l BAP and 0.1 mg/l NAA (Fig. 9).

Table 8. Effect of concentrations of BAP and NAA on the number of shoot buds formed per shoot tip explant.

Concentration of BAP (mg/l)	Concentration of NAA (mg/l)	Number of Shoot buds formed per explant
0.1	0.01	4-5
0.5	0.01	5-6
1.0	0.01	10-12
0.1	0.10	3-4
0.5	0.10	4-5
1.0	0.10	8-10

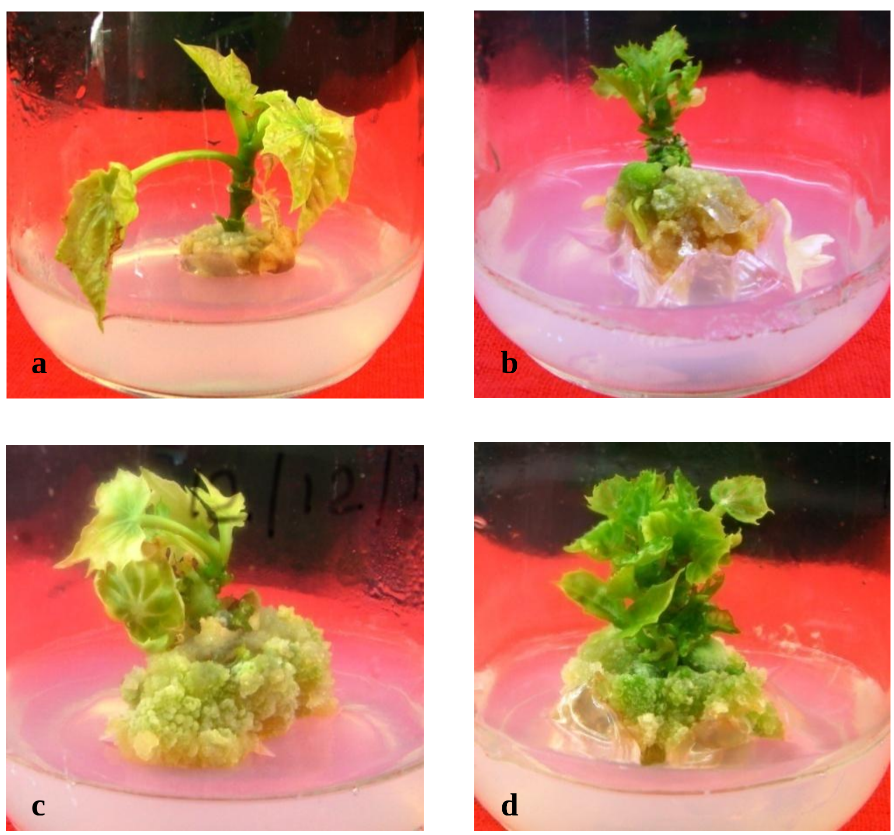


Fig. 9 Increase in number of shoots per plant with increase in BAP concentration. **a** Shoot tip cultured on MS + 0.1 mg/l BAP + 0.01 mg/l NAA. **b** Shoot tip on MS + 0.25 mg/l BAP + 0.01 mg/l NAA. **c** Shoot tip on MS + 0.5 mg/l BAP + 0.01 mg/l NAA. **d** Shoot tip on MS + 1 mg/l BAP + 0.01 mg/l NAA.

The size of the callus was also found to increase with the increase in BAP concentrations. The effect of concentration of auxin (NAA) on shoot bud formation was also analyzed. It was observed that with the increase in auxin (NAA) concentration, the number of shoot buds per plant decreased. Along with this size of callus also reduced with the increase in auxin concentration. Hence we can say that an optimum ratio of cytokinin to auxin is essential for proper shoot bud formation. The following figure (Fig.10) shows the effect of increase in concentration of NAA on the frequency of shoot buds formation and size of the callus formed per explant.

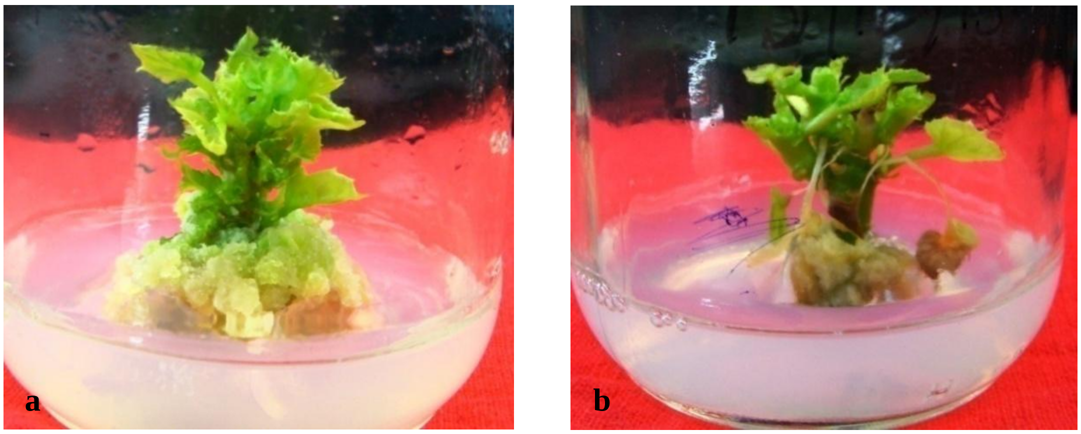


Fig. 10 Effect of NAA concentration on the number of shoot buds and the size of callus formed per plant**. a** 10-12 shoot buds formed on MS + 1 mg/l BAP + 0.01 mg/l NAA**. b** 6-8 shoot buds formed on MS + 1 mg/l BAP + 0.1 mg/l NAA.

5.3 Regeneration from the internodal stem segments of *Jatropha curcas* L.

Internodal stem segments of *Jatropha curcas* L. were brought from TJS-01#04 grown in the fields of Thapar University, Patiala. They were firstly surface sterilized by the protocol given in 4.2.6. Then under the laminar air flow hood they were cut into small pieces of about 1 inch. Fresh cuts were made on both the ends so as to remove the dead cells. The internodal stem segments were then cultured on MS medium supplemented with 0.5 mg/l BAP + 0.5 mg/l TDZ + 0.1 mg/l IBA for regeneration (Fig. 11). Results were noted after about 4 weeks. Few small shoots were found to emerge from the surface and the ends of the internode. Little callusing was also observed on the ends of the internodes.

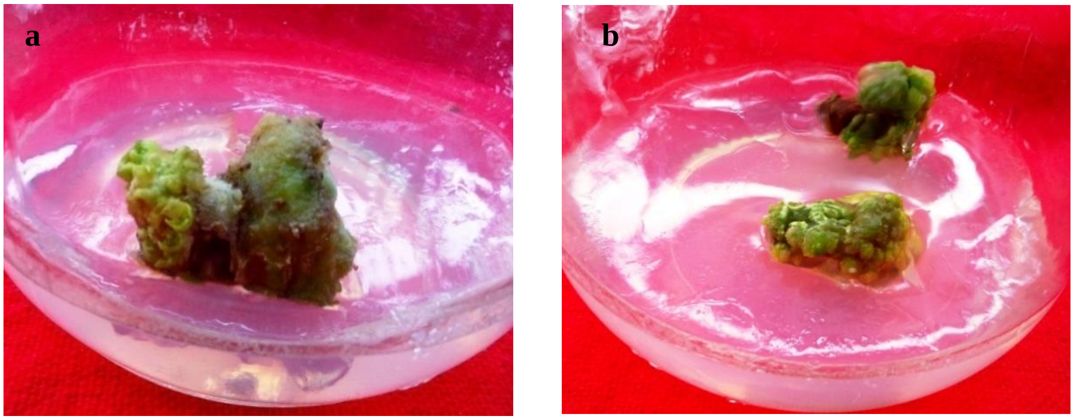


Fig. 11 a,b Shoot bud regeneration from the inter nodal segment cultured on MS + 0.5 mg/l BAP + 0.5 mg/l TDZ + 0.1 mg/l IBA.

The work is still under progress, these shoot buds are expected to grow more and then are to be shifted to the shoot elongation medium containing BAP, IBA and GA_3.

5.4 Callus formation from the leaf explants

a. Leaf explants excised from field grown plants:

Fresh leafs were brought from 2 different field plants TJS-01#04 and TJS-23#17 grown in Thapar university fields. Young soft leafs were chosen as they are very active proliferators. The leaves were surface sterilized using the protocol 4.2.6. Proper treatment with Sodium hypochlorite (5 %) for 5 minutes, bavistin (0.2 mg/l) for 5 minutes, and mercuric chloride $HgCl_2$ (0.2 mg/l) for 5 minutes was given to the explants. Under the laminar air flow hood, each leaf was cut off from both sides and both ends leaving a central rectangular piece with the midvein running through it. Then the rectangle was further cut into smaller pieces of about 1 x 1 cm perpendicular to the midvein. Different media formulations were made and were cultured with these wounded leaf explants for the callus formation.

The leaf segments were cultured on simple MS basal medium as a control. Results were observed after 3-4 weeks. No growth was observed and the leaf explants turned pale yellow to brown. For callus formation different auxin and cytokinin combinations were prepared and cultured with leaf segments. Cytokinins like BAP, TDZ were used in combination with auxins 2,4-D and IBA.

Firstly, leaf explants were cultured on MS basal medium supplemented with BAP and 2,4-D in various concentrations ranges from 0.1-0.5 mg/l. Green intact callus was observed. The rate of callus formation increased with increase in concentration of 2,4-D. After 3 weeks largest callus was observed on media containing 0.5 mg/l BAP+0.5 mg/l 2,4-D.

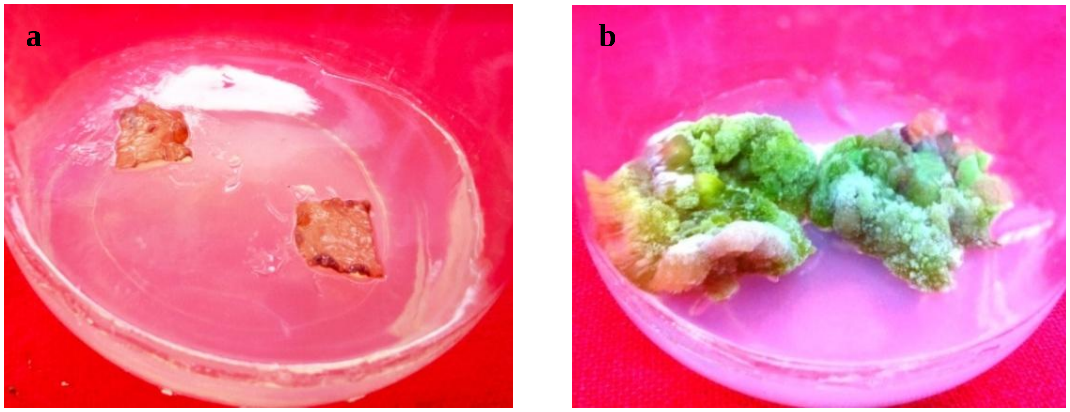


Fig. 12 Effect of 2,4-D on callus induction from field grown leafs. **a** Leaf explant on MS medium turned pale. **b** Callus induction from leaf explants cultured on MS medium + 0.5 mg/l BAP + 0.5 mg/l 2,4-D

Leaf explants were cultured on MS medium containing TDZ alone and in combination with BAP and IBA. When cultured on MS medium containing 0.5 mg/l TDZ the leaves showed swelling with little callus formation at the periphery after 3 weeks. The Leaf explants were also cultured on MS medium containing 0.5 mg/l BAP, 0.5 mg/l TDZ and 0.1 mg/l IBA (Fig. 13). Similar results were observed, the leaf explants swelled with callus formation at the peripheral areas expected to regenerate (Deore and Johnson 2008). The results are still awaited.

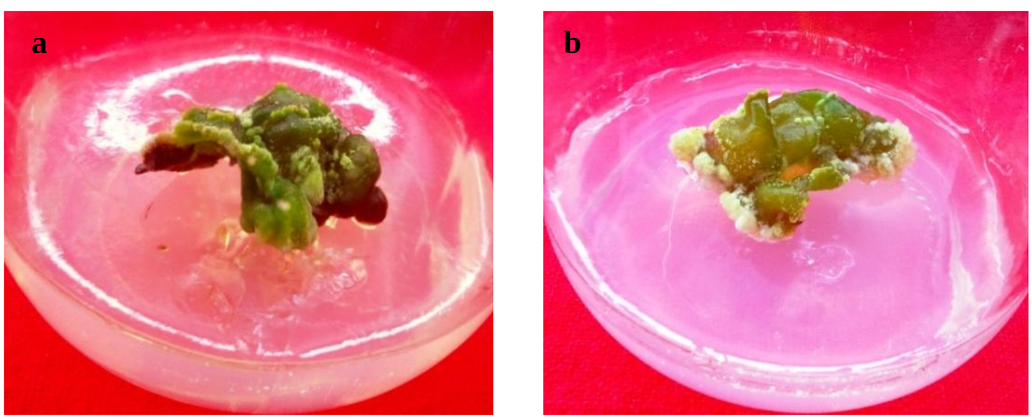


Fig. 13 Effect of TDZ on leaf explants **a** Swelling of leaf when cultured on MS + 0.5 mg/l BAP + 0.5 mg/l TDZ + 0.1 mg/l IBA. **b** Swelling of leaf on MS + 0.5 mg/l TDZ.

b. Leaf explants excised from *in vitro* grown embryo raised plants:

A very healthy, intact and green colour callus was obtained by culturing the cotyledonary leaf explants from the *in vitro* embryo raised plant on the MS basal medium supplemented with 0.5 mg/l BAP + 0.5 mg/l 2,4-D as shown in Fig. 14. It was observed that the life of callus excised from *in vitro* leaves is longer than that of callus derived from field grown leaves. Callus from field gown leaves started browning after 6 weeks whereas the callus from *in vitro* leaves remained intact and green.

Fig. 14 Effect of 2,4-D on callus formation from *in vitro* raised leaves when cultured on MS medium fortified with 0.5 mg/l BAP + 0.5 mg/l 2,4-D

5.5 Indirect organogenesis (Regeneration from callus)

Various trials were made so as to regenerate shoots from callus made in laboratory using various concentrations of BAP, TDZ and 2,4-D. The best callus was found on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l 2,4-D. Fresh green callus from about 3-4 weeks culture was chosen for the process of regeneration. The callus was cut into small pieces under the laminar air flow hood and shifted to different mediums formulated for the regeneration of callus as per literature.

The callus from MS medium containing 0.5 mg/l BAP and 0.5 mg/l 2,4-D was cut into small pieces and was shifted to MS basal medium containing TDZ in range 0.1 mg/l to 0.5 mg/l. It was observed after 3 weeks that the callus grew in mass but no regeneration was observed (Fig. 15). No shoots were observed on any of the medium. Callus grew best in MS medium containing 0.5mg/l TDZ and minimum in MS medium containing 0.1 mg/l TDZ. TDZ alone in MS medium might not be sufficient for inducing regeneration in the callus.

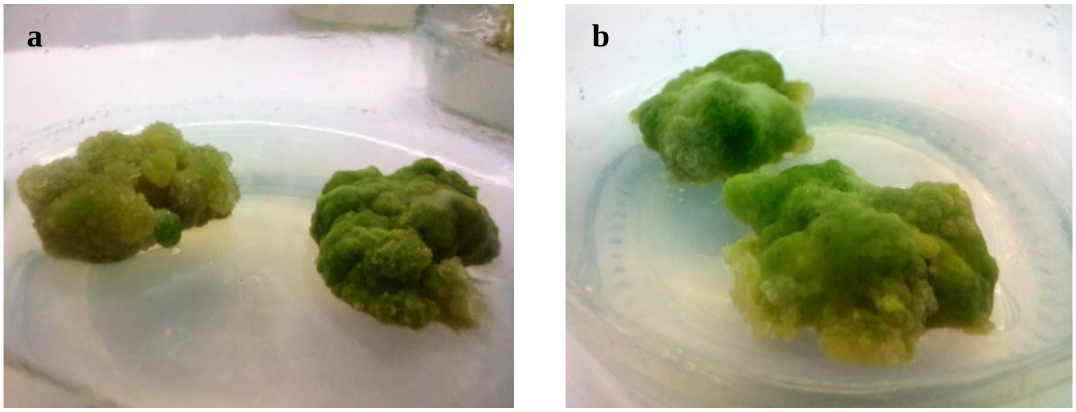


Fig. 15 Effect of TDZ on callus regeneration. **a** Callus on MS + 0.3 mg/l TDZ. **b** Callus on MS + 0.5 mg/l TDZ.

As per literature callus is expected to regenerate on MS medium supplemented with various concentrations of BAP and IBA. Hence for regeneration, the callus was shifted to MS medium containing BAP alone and various combinations of BAP and IBA. Callus on simple MS was kept as an control. In case of control the callus had not shown any response and finally died after turning pale.

First the callus was shifted to MS medium containing 0.5 mg/l BAP alone. No response was observed. Callus initially turned pale and finally brown after 4 weeks. The callus was then shifted to MS medium containing 0.5 mg/l BAP and 0.1 mg/l IBA. Callus grew in size but no regeneration was observed. Further the callus was shifted to MS medium containing 0.5 mg/l BAP and 0.5 mg/l IBA. The callus grew into a huge mass greater in size as compare MS medium containing 0.5 mg/l BAP and 0.1 mg/l IBA (Fig. 16 and Fig. 17). Thus it signifies the role of auxins in callus formation.

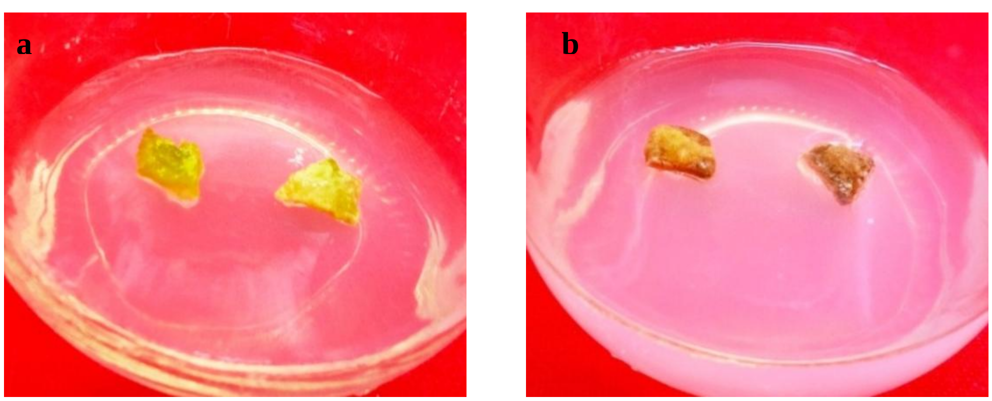


Fig. 16 Effect of BAP and IBA on callus regeneration. **a** Freshly cultured callus on MS + 0.5 mg/l BAP**. b** 4 weeks old callus on MS + 0.5 mg/l BAP.

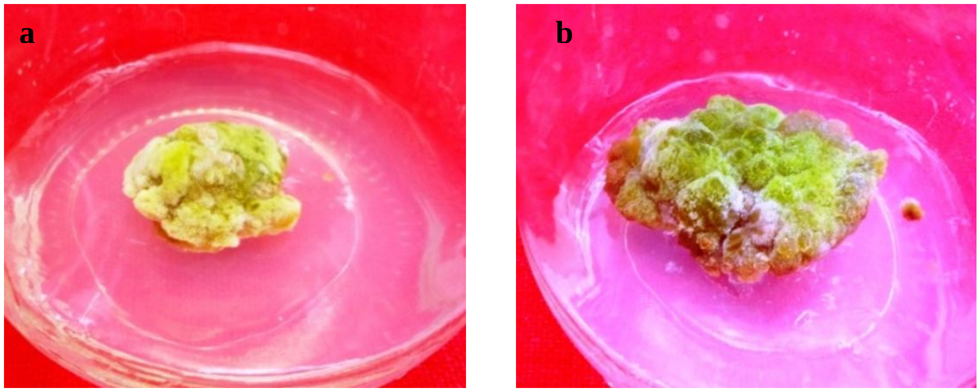


Fig. 17 a 4 weeks old callus on MS + 0.5 mg/l BAP + 0.1 mg/l IBA **b** 4 weeks old callus on MS + 0.5 mg/l BAP + 0.5 mg/l IBA.

Since no regeneration was observed on concentrations of BAP upto 0.5 mg/l, the callus from 0.5 mg/l BAP and 0.5 mg/l 2,4-D was shifted to much higher concentrations of BAP (1-5 mg/l) and IBA (0.5 mg/l). It was observed that the callus grew in mass with increase in concentration of BAP from 1 mg/l to 2 mg/l but started decreasing from 3 mg/l to 5 mg/l (Fig. 18). The callus is expected to regenerate in further days. The results are still under process.

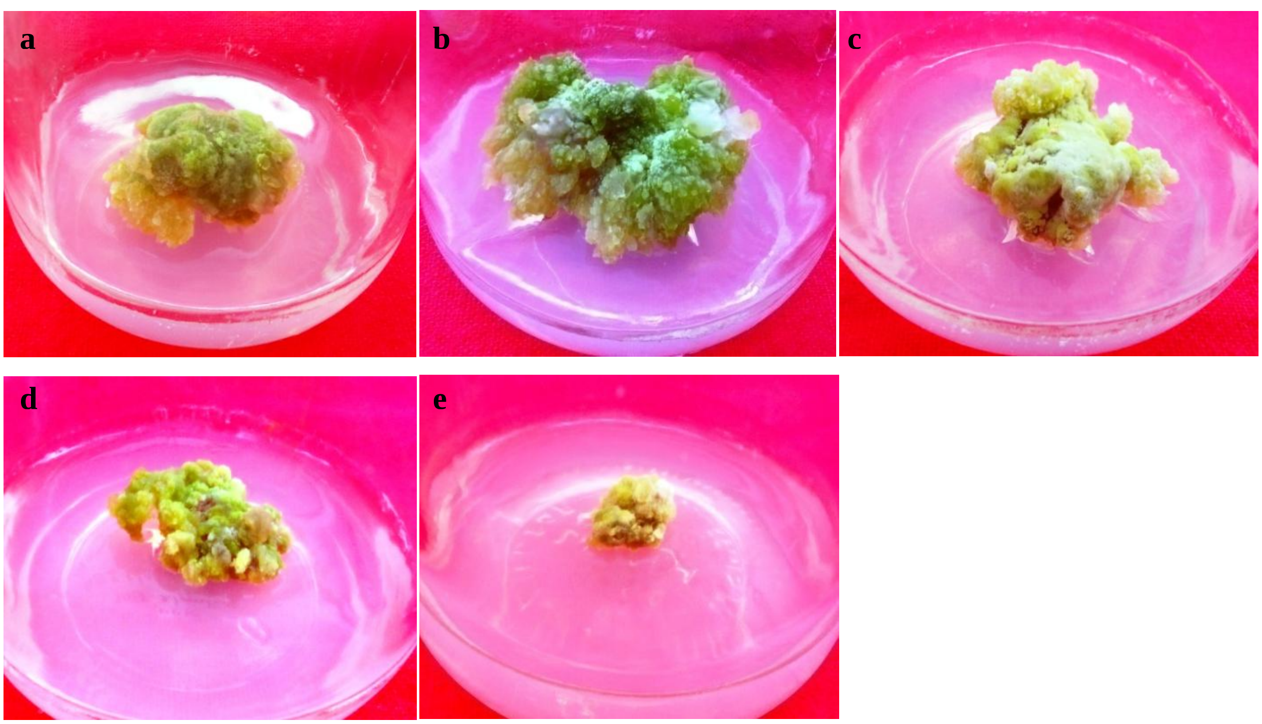


Fig. 18 Effect of higher concentrations of BAP and IBA on shoot regeneration from the callus**. a** Callus on MS + 1 mg/l BAP + 0.5 mg/l IBA. **b** Callus on MS + 2 mg/l BAP + 0.5 mg/l IBA**. c** Callus on MS + 3 mg/l BAP + 0.5 mg/l IBA. **d** Callus on MS + 4 mg/l BAP + 0.5 mg/l IBA. **e** Callus on MS + 5 mg/l BAP + 0.5 mg/l IBA.

5.6 *Agrobacterium*-mediated genetic transformation of *Jatropha curcas* L.

The internodal stem segments and leaf explants from the *Jatropha curcas* accession TJS-01#04 were sterilized using a method mentioned in section 4.2.6. Then under the laminar air flow hood, explants were co-cultivated with the *Agrobacterium tumefaciens* strain LBA4404 containing plasmid pBI121 having kanamycin resistant gene as per protocol 4.2.8. After co-cultivation the internodal stem segments and the leaf explants were cultured on MS + 0.5 mg/l BAP + 0.5 mg/l TDZ + 0.1 mg/l IBA + 80 mg/l kanamycin + 250 mg/l cefotaxime for regeneration. The results were obtained after 4 weeks. For successful transformation the explants were expected to regenerate on the medium containing kanamycin and cefotaxime.

a. Transformation of leaf explants

In case of leaf explant swelling of leafs was observed after a week. But the swelled explants very soon turned pale and died but no callusing phase was observed (Fig. 19). Hence there is a need for some improvement in the transformation protocol for the leaf explants. The work for its improvement is under the progress.

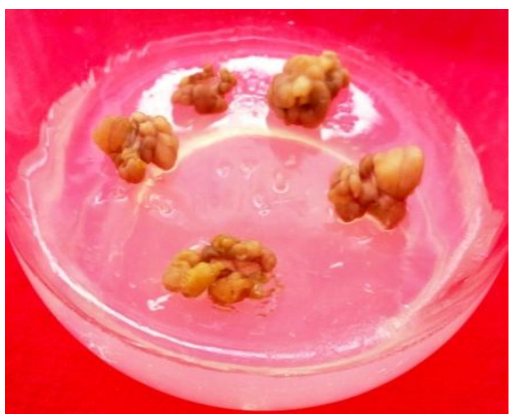


Fig. 19 Leaf explants after *Agrobacterium*-mediated genetic transformation.

b. Transformation of internodal stem segments

As per our result in section 5.3 we know that the internodal stem segments regenerate well on MS + 0.5 mg/l BAP + 0.5 mg/l TDZ + 0.1 mg/l IBA, but die when kanamycin is added to the media because they are sensitive to kanamycin. After transformation it was expected that the internodal stem segments must have gained kanamycin resistance from the *Agrobacterium* plasmid pBI121 and can now regenerate on the MS medium containing kanamycin and cefotaxime.

As a control some internodal stem segments without co-cultivation were grown on the MS media containing 0.5 mg/l BAP, 0.5 mg/l TDZ, 0.1 mg/l IBA, 80 mg/l kanamycin and 250 mg/l cefotaxime. The results for the regeneration of internodal stem segments co-cultivated with *Agrobacterium* strain were recorded after 3 weeks. It was observed that in case of control i.e. when no co-cultivation is done, the internodal stem segments have not shown any response and started turning pale. Whereas in case of internodal stem segments co-cultivated with *Agrobacterium* little callusing on the ends of the internode was observed (Fig. 20). This callus is expected to regenerate after some time. The results are still in process.

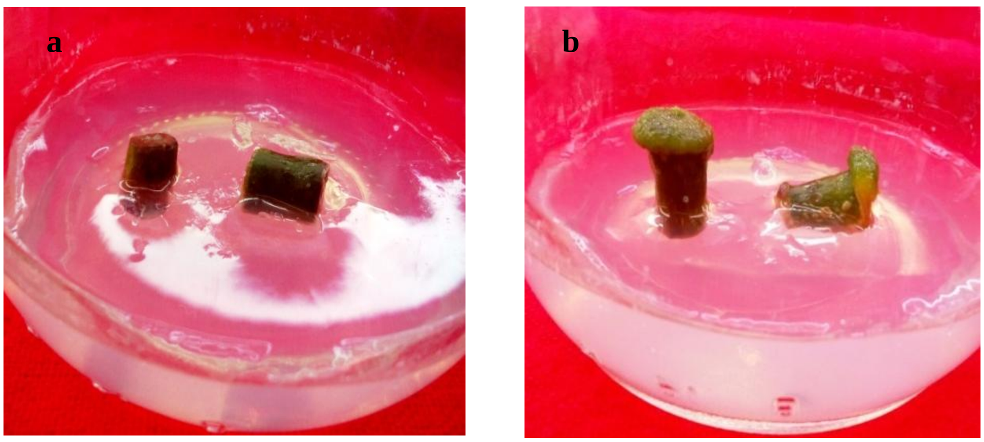


Fig. 20 Internodal stem segments transformed with *Agrobacterium tumefaciens*. **a** Internodal stem segment without co-cultivation on MS + 0.5 mg/l BAP + 0.5 mg/l TDZ + 0.1 mg/l IBA + 80 mg/l Kanamycin + 250 mg/l cefotaxime. **b** Callusing on internodal stem segments after co-cultivation with *Agrobacterium*.

Kumar et al. (2010) has reported a protocol for the stable genetic transformation of *Jatropha curcas* via *Agrobacterium tumefaciens*-mediated gene transfer using the leaf explants. But in our case the same results could not be replicated may be due to some genetic variablilities.

CHAPTER 6

CONCLUSION

All the observations and conclusions made in this study are briefly discussed below:

- Sensitivity of *Jatropha curcas* L. for various antibiotics was tested on the embryo-raised plantlets and it was found that *Jatropha curcas* was resistant to various antibiotics such as amoxicillin, ampicillin, cefoperazone, cefotaxime, chloramphenicol, hydrocortisone sodium succinate, rifampicin and tetracycline, and sensitive to amikacin, ciprofloxacin, ceftriaxone, gentamicin, ofloxacin, kanamycin, pantoparazole, and streptomycin.
- Micropropagation was partially achieved using shoot tips excised from field-grown plants. Shoot tips were found to proliferate best on MS medium containing 1.0 mg/l BAP and 0.01 mg/l NAA, with an intervening callusing. It was also observed that with the increase in auxin concentration the shoot bud regeneration tendency of the shoot tip decreases. Hence an optimum cytokinin-auxin ratio is must for proper proliferation.
- Direct regeneration of *Jatropha curcas* was found to be initiated using internodal stem segments and leaf explants excised from the field-grown plants. Internodal stem segments were found to regenerate best so far on the MS basal medium containing 0.5 mg/l BAP, 0.5 mg/l TDZ and 0.1 mg/l IBA.
- Indirect organogenesis was partially achieved using leaf explants. Firstly the callus was induced in leaf explants (both *in vitro* leafs and field grown leafs). Maximum callus was obtained on MS medium containing 0.5 mg/l BAP and 0.5 mg/l 2,4-D. This callus was then subjected to grow on regeneration medium for regeneration.
- Various regeneration media were formulated for the successful regeneration from callus. MS medium was supplemented with a very broad range of BAP (0.1-5 mg/l) and IBA (0.1-0.5 mg/l) concentrations. Maximum size of callus was observed with the increase in BAP concentration upto 2 mg/l. The callus on MS medium containing 2 mg/l BAP and 0.5 mg/l IBA is expected to regenerate very soon. The results are still awaited.
- Attempts were made for *Agrobacterium tumefaciens*-mediated genetic transformation of *Jatropha curcas* using leaf explants and internodal stem segments. Leaf explants after co-cultivation turned pale; whereas, internodal stem segments started showing callus at the ends, and are expected to regenerate on the selection medium. Further efforts are still required for standardization of genetic transformation protocol.

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