# Studies on genetic diversity and micropropagation of elite clones of *Jatropha curcas* L.

A

Thesis submitted

In the partial fulfilment of the requirements for the award of degree of

**DOCTOR OF PHILOSOPHY**

IN

**BIOTECHNOLOGY**


Submitted By

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Under the supervision of

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**February 2020**

DEDICATED TO …

MY PARENTS

(Gurbir Singh & Yogesh Rani)

# CERTIFICATE

Certified that the thesis entitled **"Studies on genetic diversity and micropropagation of elite clones of *Jatropha curcas* L."** submitted by Mr. Rajneesh Kumar, Reg. no. 901000009 in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy in the Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, Punjab is a record of candidate's own independent and original research work carried out by himself under my supervision and guidance. The material embodied in this thesis has not been submitted in part or full to any other University or institute for the award of any degree.

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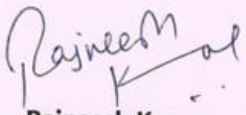
Date: 19-02-2020

## CANDIDATE'S DECLARATION

I, hereby declare that the work presented in the thesis entitled **"Studies on genetic diversity and micropropagation of elite clones of *Jatropha curcas* L."** in the partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy at Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala is an authentic record of my work during the period from January 2012 to February 2020, under the supervision of Prof. N. Das, Professor, Department of Biotechnology, Thapar Institute of Engineering & Technology, Patiala, Punjab. This report has not been submitted for the award of any degree or certificate in this or any other university.



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Rajneesh Kumar

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# List of Abbreviations

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| µL | Microlitre |
| µmol | Micro mole |
| 1H NMR | Proton Nuclear Magnetic Resonance Spectroscopy |
| AdS | Adenine sulfate |
| ANOVA | Analysis of variance |
| B-5 | Biodiesel (5 %) + diesel (95 %) |
| B-10 | Biodiesel (10 %) + diesel (90 %) |
| B-20 | Biodiesel (20 %) + diesel (80 %) |
| BAP | 6-Benzylaminopurine |
| Btu | British thermal units |
| CO | Carbon monoxide |
| CPT | Candidate plus tree |
| CRD | Completely randomized design |
| FAC | Fatty acid composition |
| FAME | Fatty acid methyl esters |
| FFA | Free fatty acid |
| FYM | Farmyard manure |
| GC | Gas chromatography |
| GCV | Genotypic coefficient of variation |
| GHGs | Greenhouse gases |
| g | Gram |
| h | Hour |
| HCl | Hydrochloric acid |
| $HgCl_2$ | Mercuric chloride |
| IAA | Indole-3-acetic acid |
| IBA | Indole-3-butyric acid |
| IC | Indigenous Collection |
| KHz | Kilohertz |
| Km | Kilometre |
| Kn | Kinetin |

| | |
|---|---|
| KOH | Potassium hydroxide |
| L | Litre |
| M | Metre |
| mg/L | Milligram per litre |
| $MH_Z$ | Megahertz |
| min | Minute |
| mL | Millilitre |
| mM | Milli molar |
| MS | Murashige and Skoog |
| NaCl | Sodium chloride |
| NOA | β-naphtoxyacetic acid |
| PCV | Phenotypic coefficient of variation |
| PGRs | Plant growth regulators |
| pH | Potential of Hydrogen |
| RH | Relative humidity |
| SD | Standard deviation |
| Sec | Second |
| SFP | Spectral flux photon |
| $SO_x$ | Sulphur oxides |
| SPSS | Statistical product and services |
| TAG | Triacylglycerol |
| TBOs | Tree borne oil seeds |
| TDZ | Thidiazuron |
| v/v | Volume per volume |
| w/w | Weight/weight |

# Abstract

Massive anthropological activities such as rapid urbanization, industrialization, huge transportation lead to depletion of conventional and non-renewable fossil fuels on our planet and also significantly compromise the environmental health through the emission of greenhouse gases (GHG). This explains why eco-friendly biofuels namely bio-diesel and bio-ethanol have become focus areas of active research during the last few decades. Some of the important perennial non-edible tree borne oilseeds (TBOs) in the Indian subcontinent include Neem (*A. indica* A. Juss), Karanj (*P. pinnata* L. Pierre), Mahua (*M. indica* J.F. Gmel), Jatropha (*Jatropha curcas L.*) which produce seed oils suitable for biodiesel production. *Jatropha curcas* L. or physic nut, a member of the *Euphorbiaceae* family, is a multipurpose deciduous small tree or shrub is now distributed in many tropical and subtropical regions of Africa and Asia. *J. curcas* draws the attention of many researchers for their rapid growth, easy propagation, drought tolerance, pest resistance, and, most importantly, high seed yield and oil content, which are prerequisites for the quality biodiesel production. A thorough survey led to select a total of 31 morphologically superior candidate plus trees (CPTs) of *J. curcas* from different locations of Punjab, a North-Western state of India. The seed samples showed considerable variation with regard to shape, size and color of the seed coats. 100-seed weight ranged from 35.10–77.34 g. Seed oil content was found to vary from 13.74% to 54.37%. Most of the accessions showed 30–40% oil content, and a few accessions showed more than 40% oil content. *J. curcas* accessions having more than 30% seed oil content and seed yield approximately 1-2 kg/tree could be referred to as elite accessions consistent with operational guidelines of DBT, Govt. of India. Both genotypic and phenotypic variances were highest and comparable for 100-seed weight followed by seed oil content. Genotypic coefficient of variation and phenotypic coefficient of variation also showed similar pattern. The highest heritability of 99% was recorded for 100-seed weight followed by oil content (97%) and seed length (81%). Positive and significant correlation was observed between 100-seed weight and oil content (r = 0.517). The *J. curcas* accessions were distinctly grouped into 6 clusters on the basis of non-hierarchical *K*-Means cluster analysis. Out of 31 candidate plus trees (CPTs), 19 *J. curcas* accessions were studied for seed oil extraction, oil quality analysis and fatty acid composition. Most of the accessions showed more than 30% oil content with free fatty acid content ranging from 0.21–1.82%. The oil samples were transesterified efficiently to fatty acid methyl esters as evident from proton nuclear magnetic resonance ($^1$H NMR) spectra. As revealed by gas chromatography, the contents of the four major fatty acids were found to

significantly vary in the seed oils viz palmitic acid (8.64–17.05%), stearic acid (4.34–7.94%), oleic acid (26.26–46.36%) and linoleic acid (28.72–53.78%). A number of seed oils showed high level of oleic acid (40.02–46.36%), and some other oil samples were rich in linoleic acid (~45%). A simple and reproducible micropropagation protocol was adopted for *in vitro* clonal propagation of elite *J. curcas* germplasm through axillary shoot bud proliferation from nodal segments. MS medium supplemented with 2.0 mg/L BAP was found to be most effective in terms of percentage explant response (95.56 ± 3.72%), number of shoot buds (1.84 ± 0.06) and shoot length (1.32 ± 0.05 cm) per explant. MS media supplemented with 0.25–2.0 mg/L of TDZ resulted in swelling at cut ends of nodal and shoot tip explants with the only formation of a bunch of condensed adventitious shoot buds; no elongation was noticed by further sub-culturing on a medium supplemented with BAP alone or in combination with IBA. Multiplication of *J. curcas* shoot buds (8.66 ± 0.21 shoots of 2.21 ± 0.04 cm length per nodal segment) was carried out by repeated transfer and sub-culture of the nodal segments produced *in vitro on* MS medium supplemented with BAP 0.5 mg/L and IBA 0.25 mg/L. For rooting purpose, ½ strength MS supplemented with 3.0 mg /L IBA effectively worked in this study. In this media formulation, 75.12 ± 4.33% shoots responded and produced 3.38 ± 0.09 roots of 4.29 ± 0.09 cm length. The *in vitro* raised rooted *J. curcas* plantlets were acclimatized by slow and gradual exposure from high RH and low-temperature conditions to low RH and high-temperature conditions. The elite *J. curcas* accessions as characterized in this study, will be useful in terms of germplasm exchange, mass propagation, multilocation trials, biodiesel feedstocks, other industrial uses, and importantly as prebreeding materials for genetic improvement of this bioenergy crop. Moreover, development of a simple, efficient and true-to-type *in vitro clonal* propagation protocol is promising to produce the quality planting materials for field trials and large scale cultivation.

Chapter 1

# Introduction

## 1.1Biofuels: An Overview

Globally, rapid urbanization, industrialization, massive transportation, and many other man-made activities not only lead to the depletion of conventional and non-renewable fossil fuels but also significantly compromised environmental health through the emission of various greenhouse gases (GHGs). The import of crude petroleum-based fuels in many countries is one of the most important factors causing a huge burden on foreign currency resrve. Therefore, there is an urgent need to explore various non-conventional renewable fuels to overcome ecological and economic issues at both national and global level. Quality production of adequate eco-friendly biofuels namely biodiesel and bioethanol have become major focus areas of active research during the last few decades (Agarwal 2007; Mukherjee et al. 2011). Various biofuels are classified into the following categories depending on the source of raw materials.

### *1.1.1 First Generation Biofuels*

Biofuels based on the edible oils from crops such as corn, wheat, soybean, peanuts, maize, sunflower, safflower, sugarcane, brassica, oil palm are usually known as first-generation biofuels (Pinto et al. 2005; Dembribus 2006).

### *1.1.2 Second Generation Biofuels*

Second-generation biofuels are based on both lignocellulose and biodiesel feedstocks:

*Lignocellulose feedstocks:* Chemically, lignocellulose biomass is composed of lignin, cellulose and hemicellulose and can act as raw materials to produce biofuels (Bhatia et al. 2017). During processing, they are converted to sugars by various thermochemical and biological processes, and finally to bioethanol through fermentation. Various lignocellulosic feedstocks are mainly agricultural residues from crops like rice, wheat, corn, sorghum, barley, and sugarcane; forest residues like logging remains, fuelwood extracts and primary and secondary wood processing milling residues. However, biofuel production using these resources has limited value due to the high cost of transportation, the operational cost of logging/collection activities, less recovery in harvest areas; and importantly herbaceous and woody energy crops. Production of biofuels from residue-based feedstocks is advantageous as they show less competition for land use and thus have a minimal direct impact on food prices (Perlack et al. 2005, Richardson 2008; Miguel et al. 2011). Major lignocellulosic feedstocks are as follows: *Energy crops*: They are non-edible energy crops with a potential to act as feedstock for biofuel production. They can be utilized as a) solid biomass

for power plants, b) gas biomass to supplement biogas production, and c) liquid biomass during its processing into liquid fuels. They are broadly classified into grassy (herbaceous or forage) and woody (tree) energy crops (Bhatia et al. 2017).

*Perennial forage crops*: In this category, the species like *Panicum virgatum* (switchgrass) and Miscanthus are considered as a promising feedstocks for biofuel production. Switchgrass has an intrinsic property of low water/nutrition input requirements, low costs, and adaptability to low-quality land. It also has a positive impact on the environment as it reduces soil erosion, improves soil health and provides habitat to wildlife. Miscanthus has cold tolerance and low nitrogen requirement properties and propagated through rhizome cuttings (Keshwani and Cheng 2009).

*Woody energy crops*: Poplar, willow, eucalyptus, and toon are referred to as woody energy crops for biofuel production. Important attributes of these species are: a) high yield potential, b) wide geographic distribution and c) low nutrient input requirements, d) control soil erosion, e) improve soil nutrient properties, f) provide wildlife habitat. These crops could be grown on the marginal, non-arable lands and grazing pastures usually not suitable for conventional production of the food crops (Smeets et al. 2007).

*Biodiesel feed stocks*: Plant species like *Hevea brasiliensis* (rubber), *Calotropis gigantia* (Ark), *Pongamia pinnata* (Karanj), *Azadirachta indica* (Neem), *Boswellia avalifololata, Madhuca longifolia (*Mahua), *Calophyllum inophyllum* (Nagchampa) and *Jatropha curcas* (Ratanjot) act as non-edible crop plants for biodiesel production (Ramashas et al. 2004; Azam et al. 2005; Mandal and Mithra 2006). The biomass residues of *J. curcas* such as fruit husks, seed shells, seed cake, or kernel meal can be converted into high-quality carbon-rich bio-coal through a thermochemical process known as biomass steam processing (BSP). The bio-coal could be used as a suitable feedstock to produce energy in domestic furnaces as well as co-firing fuel in large scale power plants (Steinbrück et al. 2019)

### *1.1.3 Third Generation Biofuels*

Microalgae are photosynthetic organisms and recognized as potential sources for biofuel production because of their high oil content, produced throughout the year and relative ease of large-scale biomass production in a cost-effective manner. Moreover, they may be grown on saline water, coastal seawater, and wastewater, mostly occupying non-arable lands, which clearly shows that these processes have limited competition with conventional agriculture as they do not use

prime agricultural land. The promising biodiesel producing microalgae species are *Botryococcus braunii, Chorella vulgaris, Chlamydomonas reinhardtii, Dunaliella primolecta, Nitzschia laevis, Gracilaria, Parieochloris incise,* and *Sargassum* (Li et al. 2008; Cristi 2007; Kalscheuer et al. 2006; Giwa et al. 2018; Mofijur et al. 2019).

### *1.1.4 National and Global Scenario on Biofuels*

The Indian Government had set a target of the complete blending of 20% biodiesel with gasoline and diesel in 2017. To achieve the target, Government of India has identified 400,000 sq. km of wasteland for *J. curcas* plantations. Moreover, the Government also started two commercial projects in the States of Andhra Pradesh and Maharashtra to check the efficacy of biodiesel utilization in diesel-driven vehicles. Both Government and different institutes such as Department of Biotechnology (DBT), National Oilseeds and Vegetable Oils Development (NOVOD), Ministry of New and Renewable Energy (MNRE), National Bank for Agriculture and Rural Development Agency (NABARD), Indian Renewable Energy Development Agency (IREDA), Small Industries Development Bank of India (SIDBI) have come forward to set up biodiesel crop plantations, *J. curcas* plantations in degraded/waste lands, oil expelling/extraction units, transesterification units, and infrastructure for storage and distribution of biofuels and their utilization in the transportation and energy sectors (Reddy et al. 2008; Wani et al. 2012; Lohan et al. 2013; Gaurav et al. 2017). In 2019, the U.S. Energy Information Administration (EIA) projected that world energy consumption would grow by nearly 50% between 2018 and 2050 (https://www.eia.gov/todayinenergy/detail.php?id=41433#). Worldwide, the total biodiesel production of some countries are: United States (60 bn. L), Brazil (29.9 bn. L), Germany (4.3 bn. L), China (3.9 bn. L) and 3.6 bn. L for Argentina (Abomohra et al. 2016). Around 30–40% of the total biofuel production in the USA, many European countries and South-East Asia depend on plant edible oils extracted from soybean, rapeseed, peanut, sunflower and palm seeds for biodiesel production (Achten et al. 2008; Demirbus 2008; Gaurav et al. 2017). India can not afford to use edible oils as feedstocks for biodiesel production as there is a short-supply of the same in Indian subcontinent. Efforts are being made to genetically improve oilseed crops like *Brassica juncea* and *Sesamum indicum* to meet growing domestic demands of edible oils (Bhunia et al. 2015; Bhattacharya et al. 2012). The focus has been entirely shifted to various tree-borne oilseeds (TBOs) which grow at different locations of the Indian subcontinent namely Neem (*Azadirachta indica*), Karanj (*Pongamia pinnata*), Mahua (*Madhuca indica*), Jatropha (*Jatropha curcas*) because of the following attributes: a) all these trees are perennial and continue to yield for a few

decades, b) they grow under different agro-climatic and edaphic conditions in the Indian subcontinent, c) ease of large-scale production of raw materials i.e., oilseeds, oil extraction, and process of transesterification, d) supportive to sustainable development, energy conservation, soil quality improvement, soil carbon sequestration, environment preservation, rural development, poverty alleviation, and wasteland reclamation, e) less requirement of agricultural inputs like chemical fertilizers and pesticides. In these species, the oil content ranged from 21 to 73 % suitable for biodiesel feedstocks (Dhyani et al. 2015). Apart from TBOs, there are some unexplored salt and drought-tolerant tree species like *Balanites aegyptiaca* promising for biodiesel production (Saini et al. 2019).

## 1.2 Bioenergy crop Jatropha (*Jatropha curcas* L.)

### *1.2.1 Taxonomy and Distribution*

The genus *Jatropha* belongs to the *Euphorbiaceae* family. There are approx. 175 known species of *Jatropha* native to South America and now widely distributed in South and Central America, Mexico, and many tropical countries (Heller 1996; Mukherjee et al. 2011). To accommodate Old and New World species, Dehgan and Webster (1979) revised the subdivision made by Pax (1910) in order to distinguish two subgenera (*Curcas* and *Jatropha)* of the genus *Jatropha*, with 10 sections and 10 subsections. They postulated the physic nut {*J. curcas* L. [sect. *Curcas* (Adans.) Griseb., subg. *Curcas* (Adans.)]} to be the most primitive form of the *Jatropha* genus. The genus derives its name from Greek word jatros (doctor) and trophe (food), indicates its medicinal values. The species commonly found in the Indian subcontinent are *J. curcas, J. glandulifera, J. gossypiifolia, J. multifida, J. nana, J. panduraefolia, J. integerrima, J. villosa, J. podagrica*, *J. tanjorensis.* Among all these species, *J. curcas and J. glandulifera* are oil yielding, and others have ornamnetnal values (De Argollo Marques 2013).

### *1.2.2 Botanical Features*

*Jatropha curcas* L. (2*n*=2*x*=22) is a multipurpose deciduous small perennial tree or large shrub (Fig. 1). It grows from semi-arid to humid environmental conditions with annual rainfall ranging from 250 to 3000 mm (Achten et al. 2008; Kumar and Sharma 2008; Iiyama et al. 2013). *J. curcas* can attain height up-to 3–5 m. Under favorable conditions, it can grow up to 8-10 m, and a productive life span could as high as 45–50 years. *J. curcas* shows articulate growth with straight soft trunk, thick branchlets and shows two flowering peaks, i.e., during summer and autumn. In humid climatic regions, flowering occurs round the year (Heller 1996).

The inflorescence is axillary paniculate ploychasial cymes with unisexual monoecious flowers. Male flowers have the following attributes: calyx with 5 segments elliptic or obviate; corolla campanulate 5 lobes with a gland at the base, connate, hairy from inside exceeding the calyx. Ten stamens are arranged in 2 distinct whorls with outer five filaments free, the inner five filaments connate in a single column in the androecium. Some features of female flowers include sepals up to 18 mm long; calyx with 5 segments as in male, corolla 4 villous inside; ovary trilocular, ellipsoid, 1.5–2.0 mm in diameter, style connate, ovules solitary in each cell. The average ratio of male to female (M/F) flowers of *J. curcas* is 22:1–29:1 in the non-native range and 60:1 in the native range (Camellia et al. 2012, Rincon-Rabanales et al. 2016). The inflorescence yields a bunch of 10 or more ovoid fruits (Fig. 2**).**

**Fig. 1.** *J. curcas* plant in the field (TJS-01#04)

**Fig. 2.** *J. curcas* plant bearing fruits (TJS-01#4)

The exocarp remains green and fleshy until the maturation of the seeds. Pollination of the physic nut occurs through insects. High fruit set in *J. curcas* indicates the prevalence of both self (32.9%) and cross-pollination (89.7%). *J. curcas* shows healthy growth on well-drained soils with good

aeration, moreover, it also grows well on gravelly, stony, sandy, calcareous and saline soils except for waterlogged areas (Heller 1996; Openshaw 2000). *J. curcas* prefers to grow in low altitude areas (0–500 m) with an average annual temperature above $20^0$ C, although it also does well even in lower temperatures with a slight frost. *J. curcas* plants start bearing fruits usually after 2 years. The maturation of seeds occurs after 2–3 months of flowering. Weight of 100-seeds among different *J. curcas* accessions varies from 35-77 g. Mature "black" seeds collected from black colored fruits with low moisture content showed higher oil yield by mechanical extraction process (Romuli et al. 2019). Under favorable environmental conditions, seed germination occurs after 10 days and the germination rate is observed best at the depth of 2-3 cm inside the soil. Seed raised *J. curcas* plants shows one tap root and 4 lateral root system. whereas, plants raised through vegetative cutting lack tap root system (Heller 1996, Negussie et al. 2015). Seeds of *J. curcas* contain toxins like phorbol esters, curcins, trypsin inhibitors, lectins and phytates making the seeds, oil and seeds cake non-edible. Curcins refer to ribosome inactivating proteins (RIPs).

### *1.2.3 Importance of Jatropha as biodiesel feedstocks*

*J. curcas* draws the attention of many researchers for their rapid growth, easy propagation, drought tolerance, pest resistance, and most importantly high seed yield and oil content which are prerequisites for biodiesel production (Heller 1996; Openshaw 2000; Fairless 2007; Mukherjee et al. 2011, Kumar and Das 2018). As evident in the earlier reports the traits such as seed yield, oil content and, oil quality vary significantly between the *J. curcas* accessions. These traits are influenced by genotype, eco-geographic conditions, agronomic practices, and various stresses (Divakara et al. 2010; Srivastava et al. 2011; Quinn et al. 2015). *J. curcas* can produce seeds containing 5-55 % oil content with an annual seed yield of 0.1 to 15 tons/ha/yr. According to the operational guidelines of DBT, Govt. of India, *J. curcas* accessions having more than 30% seed oil content and seed yield approximately 1-2 kg/tree could be referred to as elite accessions (Swarup and Munshi 2006).

### *1.2.4 Jatropha as a Multipurpose Crop*

Various uses of *J. curcas* are presented in Fig. 3.

*As a feedstock for biodiesel*: *J. curcas* is commonly regarded as a biodiesel/bioenergy crop. Biodiesel produced from its seed oil can be blended in varying proportions (B–5 to B–20) with non-renewable petro-fuels without major modifications of the combustion engines.

*As a hedge or living fence*: *J. curcas* is cultivated as an excellent bio-fence plant in most parts of India to protect agricultural fields against damage by livestock due to its unpalatable nature to cattle and goats (Jones and Miller 1992).

*As green manure*: Seed cake/press cake produced after transesterification of *J. curcas* oil is rich in nitrogen, phosphorous and potassium (NPK) and thus have a potential to act as a bio-fertilizer and can be used as feedstock for biogas production (Staubmann et al. 1997; Gubitz et al. 1999).

*As food:* A non-toxic variety of *J. curcas* from Mexico and Central America do not contain toxic phorbol esters, thus can be used for human consumption after roasting of the nuts and their deoiled high protein seed cake for feeding livestock (Makkar et al. 1997a; King et al. 2009, Valdes-Rodriguez et al. 2013).

*Soap Production*: Glycerin, the byproduct of transesterification reaction during biodiesel production can be used to make soft and durable soap.

*Insecticides*: The aqueous extract from *J. curcas* oil has potential insecticidal properties against insects like cotton bollworm and pests of pulses, potatoes and corn (Kumar and Sharma 2008)

*Medicinal uses: J. curcas* seed oil has a strong purgative/laxative action and can be sued to treat skin diseases, rheumatic pain. The sap from the stem and leaves of *J. curcas* is used in healing wounds and ulcers. (Heller 1996; Rincón-Rabanales et al. 2016). The extracts from *J. curcas* roots act as an antidote for snakebite and have anti-inflammatory activity (Mujumdar and Misra 2004).

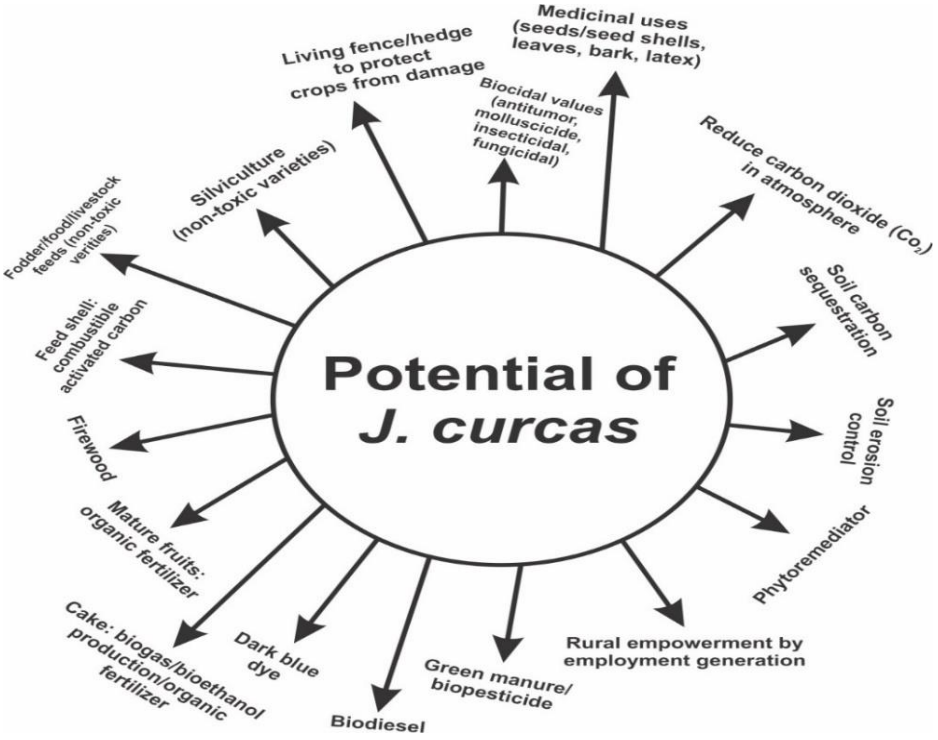


**Fig. 3.** Economic and medicinal values of *J. curcas*

Chapter 2

# Review of Literature & Objectives

## 2.1 Major Thrust Areas of Research on Jatropha

Considerable progress has been made on Jatropha research during the last more than two decades. The major thrust areas of research on this bioenergy crop are as follows:

Systematic survey, selection of candidate plus trees (CPTs), characterization and evaluation of *J. curcas* germplasm were reported for the desirable traits such as growth, morphology, reaction to major pests and diseases, seed characteristics, seed yield, oil content. Extraction of vegetable/seed oils, efficient transesterification process for biodiesel production, analysis of free fatty acid (FFA) and fatty acid composition (FAC) are also attractive aspects to the researchers and policy makers of many countries (Fukuda et al. 2001; Kaushik et al. 2007; Rao et al. 2008; Mishra 2009; Tripathi et al. 2013; Duong et al. 2013; Francis et al. 2017; Kumar and Das 2018a; Kumar and Das 2018b).

Genetic improvement was carried out to develop superior *J. curcas* genotypes with high seed and oil yield, early maturing, dwarf varieties, pistillate lines, stability of genotypes across different ecogeographic regions and resistance to biotic and abiotic stresses to meet the requirements of different stakeholders viz, researchers, farmers, commercial growers, biofuels industries along with land reclamation (Carels 2009; Divakara et al. 2010; Martin and Montes 2015; Senger et al. 2016).

Different molecular markers such as RAPD (Random amplified polymorphic DNA), AFLP (Amplified fragments length polymorphism), ISSR (Inter simple sequence repeat) and SSR (Simple sequence repeat or microsatellite) were developed for assessment of interspecific and intraspecific genetic variability, selecting diverse genotypes for hybrid development, hybrid purity testing in the elite germplasm (Prabakaran and Sujatha 1999; Basha and Sujatha 2009; Tatikonda et al. 2009; Kumar et al. 2011; Pioto et al. 2015). Ricci et al. (2012) identified the distinct *J curcas* genotypes and the population-specific molecular markers useful in breeding programs. Development of toxic and non-toxic DNA marker systems in *J. curcas* helped in growing some non-toxic *J. curcas* varieties on a large scale which could help in utilization of their seed oils for human consumption and deoiled press cake as feed for the livestocks thus adding commercial values to the crop. The marker systems will be useful in identifying the non-toxic varieties from the collection of *J. curcas* accessions (Basha and Sujatha 2007; Pamidimarri et al. 2009b).

Mass propagation, multi-location trials, performance evaluation were carried out under different agro-ecological conditions with assessment of their genetic variation and development of agro-technologies (Jingura et al. 2011; Singh et al. 2013). The seed yield

variability of the *J. curcas* accessions were influenced by various parameters such as genetic factors, age of the plants, ecogeographic conditions, soil fertility, rainfall and agronomic practices (Lama et al. 2018). Lack of availability of proper agronomic practices affects both seed and oil yield of the selected *J. curcas* accessions. Variation in seed yield and oil content between the *J. curcas* accessions sometimes misled the farmers and policymakers for large-scale cultivation of this crop. Therefore, apart from developing superior genotypes through conventional and molecular breeding, there is an urgent need to optimize agronomic practices (Srivastava et al. 2011).

*J. curcas* is mostly cross-pollinated crop. Genetic variation was assessed by the following methods: mass selection, recurrent selection, mutation, heterosis breeding, interspecific hybridization, and intraspecific hybridization (Divakara et al. 2010; Kumar et al. 2015). Analysis of the genome sequence (Sato et al. 2010), development of the molecular markers for genotyping (Basha and Sujatha 2007; Mastan et al. 2012a; Mastan et al. 2012b), proteome and transcriptome analyses, isolation and characterization of different genes, functional genomics (Yang et al. 2009; Costa et al. 2010; Maghuly and Laimer 2013) are also diverse facets of Jatropha research.

For development of novel *J. curcas* varieties with desirable phenotypes various biotechnological approaches/molecular techniques are being adopted such as micropropagation, direct and indirect organogenesis, development of simple transformation protocols, facile transgenic techniques, marker-assisted selection and breeding (Li et al. 2008; Kumar and Reddy 2010; Mukherjee et al. 2011; Rathore et al. 2015).

## 2.2 An Account of *Jatropha curcas* Germplasm

### *2.2.1 Survey, selection and characterization of Jatropha germplasm*

Extensive survey and selection of *J. curcas* candidate plus trees (CPTs) significantly contributed for evaluation of the following attributes such as morphology and physiological aspects, floral traits along with other traits such as fruit maturation, seed yield, oil content, plant height, adaptability to different climatic conditions, pest and disease resistance, branching, M/F ratio, flowering time, phenological behaviour, anthesis pattern. The yield of any crop is a direct result of proper agronomic practices that include viz, nursery development, mass multiplication of quality planting materials, spacing pattern, intercropping patterns, pruning techniques, application of PGRs, integrated water and pest management, harvesting, processing, handling, storage and viability of seeds. These parameters were considered during selection of the *J. curcas* genotypes with high seed yield, oil content with desirable fatty acid composition

(Heller 1996; Kaushik et al. 2007; Rao et al. 2008; Mishra 2009; Achten et al. 2010; Divakara et al. 2010; Tripathi et al. 2013; Duong et al. 2013; Martin and Montes 2015; Wani et al. 2016; Francis et al. 2017).

There are several reports on survey, selection, and evaluation of the *J. curcas* genotypes from different eco-geographical locations for studying growth parameters, morphology, seed characteristics and yield traits, response to pests and diseases followed by assessment of genetic variability and diversity for various desirable attributes. Usually, large collection of *J. curcas* germplasm from the candidate plus trees (CPTs), their conservation and evaluation are essential to understand variability (Kaushik et al. 2007; Rao et al. 2008; Tripathi et al. 2013). The high adaptability of *J. curcas* germplasm to a wide range of edaphic and eco-geographical regions of the world suggesting the existence germplasm variability which could be exploited for commercial purposes (Jongschaap et al. 2007; Rao et al. 2008; Behera et al. 2010). *J. curcas* seeds collected from different locations of Central India representing two states Madhya Pradesh and Maharashtra showed oil content from 33–39% (Ginwal et al. 2004). Variation in seed oil content (27.8–38.4%) and 100 seed weight (44–77 g) was observed in some Indian accessions (Wani et al. 2006). A number of *J. curcas* accessions were collected from the different agro-climatic zones of Haryana, India. They showed variability in seed traits and oil content: 100-seed weight (49–69 g) and oil content (28–39%) (Kaushik et al. 2007). Differences were observed in the *J. curcas* accessions collected from Andhra Pradesh, India: 100-seed weight (57–79 g) and oil content (30–37 %) (Rao et al. 2008). Variation in seed oil content i.e., 22–42 % was noticed in 162 collected *Jatropha* accessions growing in four distinct eco-geographic zones of peninsular India (Sunil et al. 2008). Seed oil content variation from 17–35 % was reported from 10 selected *J. curcas* accessions growing from the plantation sites at Solar Energy Centre, Gurgaon, India (Srivastava et al. 2011). Variation in the seed oil content and 100-seed weight was ranged from 20.29–61.83 % and 28.6–80 g, respectively in the collected *J. curcas* accessions from 39 provenances of the seven states of Nigeria (Halilu et al. 2011). *J. curcas* accessions collected from 18 different provenances in West and East Africa, the Americas and Asia showed variation with regard to crude protein, fat, fiber and ash contents (Makkar et al. 1997a). Progeny trial of the *J. curcas* accessions showed variation in their growth, phenological, seed and oil characteristics under tropical monsoon climatic conditions of Bhubaneswar, India (Mohapatra and Panda 2010). In the North-West part of India, second flowering during July to November usually yielded high fruit set, number of seeds per fruit and oil content (Kaur et al. 2011). Evaluation of 17 *J. curcas* accessions was

carried out in South Florida to assess floral and reproductive traits such as the number of female flowers, male to female flower ratio, fruit set, *in vitro* pollen germination, and the formation of fruits by apomixis, self-pollination, and natural pollination (Nietsche et al. 2014). Assessment of the group germplasm, sampling (single vs. multiple seeds), phenotypic variation and the associated seed traits were reported in the *J. curcas* seed samples collected from wide geographical regions (Montes et al. 2013).

### *2.2.1 Phenotypic and genotypic diversity of Jatropha*

Phenotypic diversity analyses allow reliable classification and identification of the collected *J. curcas* accessions for utilization in the specific breeding purposes. *In situ* methodology was adopted to assess the phenotypic traits of the superior *Jatropha* lines (Sunil et al. 2008). Using geographic information system (GIS), Sunil et al. (2009) developed a DIVA-GIS method based on plant height, number of primary branches, collar diameter, number of fruits per cluster and oil content. The purpose was to search the potential areas with regard to distribution pattern of germplasm with high oil content. Significant phenotypic variation was observed in seed weight and oil content of 2000 *J. curcas* seed samples collected from China, Laos, Cambodia, and Burma by Nuclear Magnetic Resonance (NMR) method (Wu et al. 2012). 34 (thirty-four) *Jatropha* cultivars from Assam were evaluated based on plant height, stem girth, branches per plant and 100-seed weight along with adaptive trials (Saikia et al. 2009). A significant difference in vegetative development except leaf shape was noticed among the 13 provenances of *J. curcas* in the multilocation trials conducted in two countries, viz. Senegal and Cape Verde (Heller 1996).

Correlation between various traits, broad-sense heritability, and genetic advance is a useful criterion for selection. The coefficient of variance between 24 *J. curcas* accessions collected from Haryana, India indicated relatively less environmental influence on the seed traits and oil content. However, seed weight and oil content showed a significantly positive correlation (Kaushik et al. 2007). High broad-sense heritability and high genetic advance for oil content in *J. curcas* accessions indicated potentiality for further improvement through selection (Rao et al. 2008, Kaushik et al. 2007). The evaluation of genetic association and variability in seed traits and growth characters was carried out; it was found that Male/Female flower ratio had the maximum positive direct relationship with seed yield (0.789), followed by number of branches (0.612), and number of days from fruiting to maturity (0.431) with high heritability for their traits (Rao et al. 2008). Likewise, seed sources collected from central part of India had shown that seed oil content was significantly correlated with seed weight (0.792); stem

diameter (0.836) and total leaf area (0.883) (Ginwal et al. 2004). *J. curcas* can thrive well in areas with 200 mm $y^{-1}$ rainfall, but it requires a minimum of 600 mm $y^{-1}$ rainfall to bear fruits (Heller 1996; Krishnamurthy et al. 2012). Seed yield and growth of *J. curcas* were found to increase with increasing rainfall up to 1500 mm $y^{-1}$, but decreased markedly with higher level of rainfall due to sensitivity towards waterlogging (Trabucco et al. 2010; Gimeno et al. 2012).

### *2.2.2 Genetic diversity analyses*

Development and use of DNA based molecular markers are significant advancements in the field of molecular genetics for detection and exploitation of DNA polymorphism. Moreover, these markers help in modern breeding programs with the establishment of molecular fingerprints for distinct and most divergent accessions using diversity analysis (Caetano-Anolles and Gresshoff 1997). Usually, in different trees species, genetic diversity is highest at native places, and low to moderate in the eco-geographical areas where it has been introduced from its center of origin (Santos et al. 2016; Laviola et al. 2017; Lama et al. 2018). In the case of *J. curcas*, genetic diversities were highest within its native range of Mexico and Costa Rica. Out of 109 studied accessions from ten countries, 92 accessions collected from Asia, Africa, and Honduras had shown a low level of genetic diversity, in comparison to 17 accessions collected from Mexico and Costa Rica which showed a high level of genetic diversity (Santos et al. 2016). Moreover, in their native range, *J. curcas* produced 84% of fruitsets through cross-pollination and 16% by self-pollination resulting in development of heterozygous genotypes (Rincón-Rabanales et al. 2016). Observation of relatively low genetic diversity of *J. curcas* germplasm in its non-native range was due to its propagation through vegetative cuttings and descendance from a smaller number of common ancestors (Biabani et al. 2013; Pamidimarri and Reddy 2014).

### *2.2.3 Interspecific genetic diversity and phylogenetic relationship*

Different types of DNA-based molecular markers such as RAPD, AFLP, ISSR, SSR remained useful for a) evaluation of genetic diversity, b) genotype characterization and identification, c) establishing the phylogenetic relationship of *J. curcas* with other related *Jatropha* species, d) selection of diverse genotypes for hybrid development and its purity testing, e) marker-assisted selection, and f) gene pyramiding (Prabakaran and Sujatha 1999; Xu et al. 2012; Kumar et al. 2015). Ganesh Ram et al. (2008) examined the genetic diversity of 12 *Jatropha* species based on the RAPD markers. Out of 26 random primers, 18 primers revealed 80.2% polymorphism across the tested set of genotypes indicating broader genetic base between wild relatives of *J. curcas.* By using RAPD and AFLP markers, genetic diversity and the phylogenetic

relationship were established between the seven *Jatropha* species viz., *Jatropha curcas, J. glandulifera, J. gossypifolia, J. integerrima, J. multifida, J. podagrica* and *J. tanjorensis*. Mean percentage polymorphism was 68.48% by RAPD and 71.33% by AFLP markers. Maximum relatedness between two species viz., *J. curcas* and *J. integerrima* suggested inter hybrid crosses between these species (Pamidimarri et al. 2009a). Molecular characterization was carried out using ISSR, RAPD and organelle-specific microsatellite primers to compare 34 Indian accessions comprising eight agronomically important species namely *J. curcas, J. gossypifolia, J. glandulifera, J. integerrima, J. podagrica, J. multifida, J. villosa.* var. *ramnadensis, J. maheshwarii* and a natural hybrid *J. tanjorensiswas*. The nuclear marker systems revealed high (98.5%) polymorphism indicating interspecific genetic variation corroborated with the morphological variations (Basha and Sujatha 2009). Three marker systems namely RAPD, ISSR, and DAMD were used for the molecular characterization of 19 *Jatropha* accessions, which included 15 *J. curcas* accessions and four different species viz., *J. podagrica, J. gossypiifolia* and *J. integerrima* and *J. tanjorensis*. The highest polymorphism (96.67%) was recorded by RAPD followed by DAMD (91.02%) and ISSR (90%) (Murty et al. 2013). RAPD and AFLP molecular markers were used to assess genetic variation in 38 *J. curcas* accessions collected from 13 countries of 3 different continents. The results showed narrow genetic diversity; 6 Indian *J. curcas* accessions showed considerable genetic diversity indicating possibilities of improvement through interspecific breeding (Popluechai et al. 2009). By using four AFPL primer combinations, genetic diversity was assessed for 6 *Jatropha* species viz., *J. curcas, J. gossypifolia, J. integerrima, J. glandulifera, J. poda- grica* and *J. dioca.* Among all the species, *J. curcas* showed highest similarity with *J. dioca* (59%) followed by *J. integerrima* (53%) (Sinha and Tripathi 2013).

### *2.2.4 Intraspecific genetic diversity and phylogenetic relationship*

Evaluation of genetic variability/diversity was carried within *J. curcas* germplasm collected from different ecogeographical areas and/or agroclimatic zones of the world by using different types of molecular markers. A worldwide analysis of *J. curcas* germplasm showed significantly higher genetic variability in the Mesoamerican population than the African and Asian populations (Li et al. 2017). Assessment of genetic variability was carried out among 42 *J. curcas* accessions collected from various agroclimatic zones of India along with a non-toxic variety from Mexico. 400 RAPD and 100 ISSR molecular markers showed 42.0% and 35.5% polymorphism respectively among the accessions which indicated the existence of low levels of genetic diversity among Indian germplasm. Introduction of accessions from broader

ecogeographical and agroclimatic regions could widen the genetic resources of *J. curcas* (Basha and Sujatha 2007). Genetic diversity was assessed by utilizing RAPD and AFLP markers for selected 23 *J. curcas* accessions showing 14-16% and 8-10% polymorphism, respectively (Reddy et al. 2007).

SSR and AFLP marker systems were employed to assess genetic variability among 58 *J. curcas* accessions collected from different geographic regions of China (Sun et al. 2008). The highest genetic variability was observed in the *J. curcas* accessions collected from Guizhou region of China. 192 Brazilian *J. curcas* accessions were examined by 6 microsatellite primers and 96 RAPD primers; the results showed that all the accessions were homozygous except one at microsatellite region (Rosado et al. 2010). Genetic diversity of the 117 Brazilian *J. curcas* accessions was assessed by using a combination of phenotypic and genotypic characters. The genetic diversity varied from 0 to 1.29, with a dissimilarity between genotypes of 0.51. Analysis of molecular and phenotypic variability reveals that the genetic diversity among the *J. curcas* accessions was 64% and 15.6% higher than the variability assessed from the phenotypic and molecular characters respectively (Alves et al. 2013). Methylation-sensitive amplification polymorphism (MSAP) technique was used to evaluate genetic diversity among 56 accessions of *J. curcas* collected from Thailand and other countries. Results revealed high genetic similarity (ranging from 0.95 to 1.00) among the studied accessions, indicating prevalence of low genetic diversity in the species (Kanchanaketu et al. 2012). Amplified fragment length polymorphism (AFLP) was employed to assess the genetic diversity in a total of forty-eight *J. curcas* accessions covering six states of India. A total of 680 (88 %) fragments showed polymorphism in the germplasm, out of this, 59 (8.7 %) fragments were unique i.e., accessions specific and 108 (15.9 %) fragments were rare i.e., present in less than 10 % accessions. *J. curcas* accessions collected from Andhra Pradesh were found to be diverse, and accessions belonging to Chhattisgarh showed the presence of a higher number of unique fragments (Tatikonda et al. 2009). Use of SSR markers was found useful for genetic diversity analysis between Indian and global germplasm, mapping populations and hybridization for future breeding and genetic improvement programs (Maurya et al. 2015). The genetic variability was found in 224 *J. cucas* accessions (219 from China and 5 from Myanmar) using 15 ISSR markers. Among the 169 amplified bands, 127 (75.15%) were polymorphic which concludes that Chinese *Jatropha* had high genetic diversity (Cai et al. 2010). Analysis of molecular diversity through RAPD was carried out for 160 *J. curcas* individual trees from 8 different populations of Kenya. Analysis of molecular variance (AMOVA) indicated distinct variability in the *Jatropha* accessions including their seed traits (Machua et al. 2011).

## 2.3 Biofuels-A Renewable Energy Source

Use of various non-conventional renewable energy sources such as solar energy, different biological resources, hydropower, wind, sea tide have become a common trend in many countries in order to meet the ever-growing energy demands. Rudolf Diesel invented the diesel engine in the 1890s. It was a significant contribution as this engine could run on a variety of fuels, including vegetable oil. The conversion of vegetable oil to fatty acid methyl esters (FAME) was reported in the 1930s in Belgium. In USA, the production of biodiesel from used cooking oil was started in 1996. In India, biodiesel production from the renewable vegetable feedstocks got boosting after 2001 due to scarcity and rise in the prices of petroleum products.

### *2.3.1 Biodiesel: A Precise Account*

The term 'Biodiesel' is defined as a fuel made from a mixture of fatty acid methyl ester (FAME) of vegetable oils (both edible and non-edible), and animal fats through the process of transesterification. During this process, triacylglycerol (TAG) molecules react with an alcohol namely methanol or ethanol in the presence of a catalyst, usually potassium hydroxide to form fatty acid methyl/ethyl esters and glycerol (Fig. 3).

Biodiesel can be produced from renewable biomass resources such as energy crops, agricultural crops and trees, edible food crops, fiber crop residues, algae, woody residues, and agricultural wastes. Biodiesel has the following desirable attributes: renewability, biodegradability, provide energy security, non-toxicity, portability, ready availability due to domestic origin, low sulphur content, excellent lubricity, higher flash point, higher cetane number, and ease of oil extraction and processing. It can be blended in varying proportions (B–5 to B–20) with non-renewable petro-fuels. In addition, the combustion of biodiesel in a conventional diesel engine reduces emissions of unburned hydrocarbons, particulate matter, carbon monoxide (CO), polycyclic aromatic hydrocarbons, soot and sulphur oxides (SOx). Relatively a new term i.e., 'renewable diesel' is coined which resembles petrodiesel produced by cracking or pyrolysis; hydrodeoxygenation is also gaining importance in the recent years (Berchmans and Hirata 2008; Fukuda et al. 2001; Knothe 2009; Knothe 2010, Openshaw 2000; Ong et al. 2011, Sajjadi et al. 2016, Giwa et al 2018).

The composition of a quality biodiesel should comply with the following parameters: the presence of high amounts of monounsaturated fatty acids (MUFA) such as C16:1 and C18:1, reduced level of the polyunsaturated fatty acids (PUFA) and optimal presence of the saturated fatty acids (Knothe 2009; Pinzi et al. 2009). Currently, biodiesel cannot entirely replace petroleum-based diesel fuel but can help in the reduction of alarming oil import bills,

strengthening the global economy, mitigating climate change, enhance environmental quality, growth in GDP, generation of jobs.

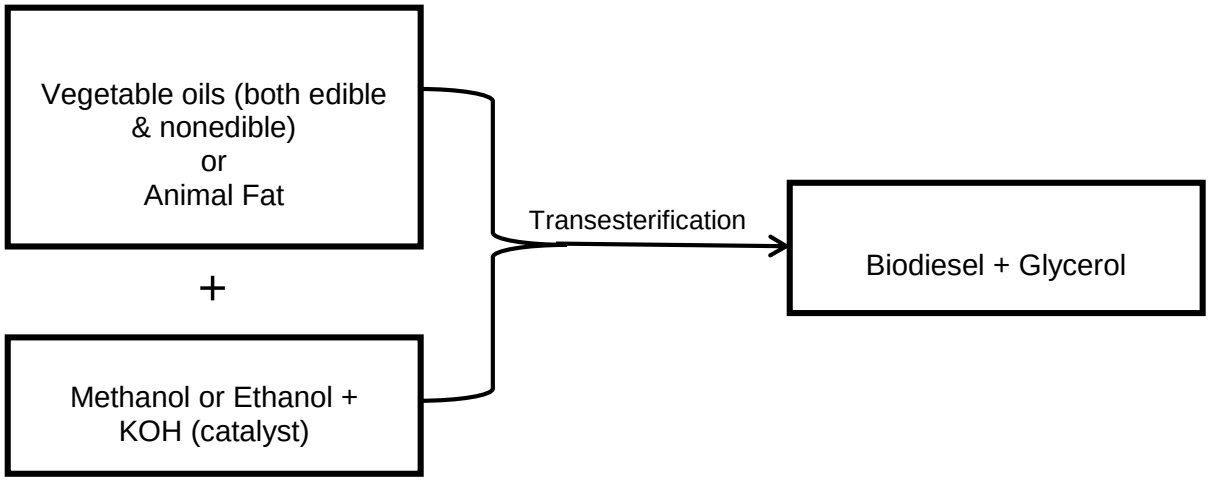


**Fig. 3.** Basic steps of Biodiesel production

*Transesterification and Fatty Acid Analysis*: As a future alternative fuel, biodiesel should meet national energy security and show economic competitiveness with petroleum-based diesel fuels. The cost of biodiesel production can be reduced by utilizing less expensive feedstocks like non-edible vegetable oils, animal fats, waste cooking oil, by-products of refined vegetable oils, industrial wastes, and municipal wastes. But the availability and sustainability of continuous supply of these feedstocks could be a limiting factor for biodiesel production and subsequent delivery at commercial oil filling stations (Bhatia et al. 2017). Therefore, large-scale production of biodiesel from various vegetable oils including *J. curcas* through transesterification has become an important area of research in many laboratories. Various parameters are known to influence the tranesterification reaction as evident from several reports. For example, oil resources and its fatty acid composition, oil quality, nature of the contaminants and their contents, methanol/ethanol-to-oil ratio, temperature, nature of catalysts, reactor/separator design, hydrodynamic conditions such as volumetric flow rates, phase ratios and catalyst concentrations and other process conditions influence the transesterfication process (Ghesti et al. 2007; Klofutar et al. 2010; Wang et al. 2011; Likozar and Levec 2014; Likozar et al. 2016). Noncommercial lipases like Sn-1,3-regioselective rROC and rCPL are being used as substitute of base catalysts in the transesterification process with high FAME yields ranged from 51-65%, monoglycerides avoiding glycerol formation as byproduct. (Rodrigues et al. 2016).

*Biodiesel quality*: Quantitatively, seed oil content is a desirable attribute of the individual *J. curcas* accessions; but the parameters like free fatty acid (FFA) content and fatty acid composition (FAC) are major influencing factors in determining the quality of a biodiesel (Tiwari et al. 2007; Wang et al. 2011). High FFA content in the oils is due to the hydrolysis of triglycerides in the presence of moisture and oxidation, which in turn has adverse effects on the transesterification process because of soap formation causing low yield of biodiesel product. FAC analysis helps to assess the fuel properties of a biodiesel such as cetane number (CN), oxidative stability (OS), viscosity, lubricity and cold flow properties (Demirbus 2008; Knothe 2009; Ramos et al. 2009). The presence of high concentrations of FFA in the crude *J. curcas* seeds reduced the yield of methyl ester formation in the alkali base-catalysed transesterification process. In these conditions, acid pre-treatment is recommended to reduce the concentration of FFA below 1%, followed by the transesterification process to produce biodiesel (Berchmans and Hirata 2008). Apart from searching the superior *J. curcas* genotypes with high seed oil content and desired FAC, some other strategies were also adopted successfully in developing the designer crops. For example, Qu et al. (2012) reported marker-free RNA interference transgenic *J. curcas* plants with significantly increased level of oleic acid in seed oil. A few *J. curcas* lines with very high oleic acid content were generated through genetic crossing (Sinha et al. 2016).

## 2.4 Micropropagation of *J. curcas*

Development of a simple, efficient, cost-effective and mass scale true-to-type micropropagation protocol is a prerequisite to produce and supply the quality planting material for large scale cultivation and field trials. There is a need of fast and large-scale multiplication of the elite *J. curcas* germplasm for cultivation in the field condition. There are several reports on the tissue culture protocols as developed for the bioenergy crop *J. curcas* (Rathore et al. 2015; Kumar et al 2015).

### *2.4.1 Direct Regeneration*

*In vitro* plant regeneration is a key tool of plant biotechnology. It works on the totipotent nature of plant cells i.e., the ability of a single cell to grow into a new plant (Haberlandt 1902). *In vitro* plants can be regenerated in two ways: direct regeneration without any intervening callus phase and indirect regeneration with intervening callus phase. Direct regeneration is a reliable

method of micropropagation and exhibits high genetic stability. The plant cells regenerate in a medium containing the nutrients and plant growth regulators (PGRs). Some commonly used growth regulators are BAP, Kn, TDZ, IBA, IAA, NOA. For *J. curcas*, almost various explants were reported for direct regeneration: leaf (Sujatha and Mukta 1996; Sujatha et al. 2005; Deore and Johnson 2008; Khurana-Kaul et al. 2010; Reddy et al. 2008; Divakara et al. 2010), shoot tips (Rajore and Batra 2005, Purkayastha et al. 2010), nodal segments (Sujatha et al. 2005; Datta et al. 2007; Rathore et al. 2015), petiole (Sujatha and Mukta 1996; Kumar and Reddy 2010; Kumar et al. 2011), hypocotyls (Sujatha and Mukta 1996; Kaewpoo and Te-chato 2009), epicotyls (Qin et al. 2004), and cotyledons (Kumar and Reddy 2010).

In toxic *J. curcas* accessions, shoot bud induction and plant development were studied by using various explants viz., leaf, hypocotyl, petioles. Organogenesis occurred successfully from the explants like hypocotyl and petiole on MS medium containing BAP and IBA (Sujatha and Mukta 1996). *In vitro* propagation of non-toxic *J. curcas* variety was developed through axillary bud proliferation and direct adventitious shoot bud regeneration from leaf segments on MS medium supplemented with 2.2-44.4 µM BAP, 2.3-46.5 µM Kinetin and 2.3-45.6 µM TDZ individually. Efficient regeneration of shoot buds from leaf tissues was found on MS medium enriched with 8.9–44.4 µM BAP and 4.9 µM IBA followed by transfer to MS medium containing 8.9 µM BAP and 2.5 µM IBA (Sujatha et al. 2005). Qin et al. (2004) reported shoot bud regeneration from epicotyl explants on MS medium containing 0.1 mg/L IBA and 0.5 mg/L BAP. Callus induced shoot regeneration was noticed on MS medium supplemented with from 1.0 mg/L IBA and 0.5 mg/L BAP. The proliferation of shoots tips was observed on MS medium supplemented with 2.0 mg/L BAP and 0.5 mg/L IAA along with adenine sulphate, glutamine, and activated charcoal. MS containing 3.0 mg/L IBA worked effectively for rooting (Rajore and Batra 2005). MS medium supplemented with 0.90 µM TDZ and 0.98 µM IBA produced adventitious shoot buds directly on the surface of the leaf explants without formation of intervening callus; while shoot bud proliferation was accompanied with callus formation on medium supplemented with 13.3 µM BAP and 2.46 µM IBA suggesting superiority of TDZ to BAP in morphogenic response (Khurana-Kaul et al. 2010). Adventitious shoot bud induction from young leaf explants was also observed on MS medium supplemented with 2.27 µM TDZ, 2.22 µM BAP and 0.49 µM IBA. The presence of TDZ in the induction medium has provided high efficiency of organogenesis of bud from leaf explants, whereas BAP without the addition of TDZ promoted callus induction rather than shoot bud proliferation (Deore and Johnson 2008). Prior treatment of the petiole explants with high concentrations TDZ

(5–120 mg/L) for a short period (5–80 min) significantly enhanced the regeneration frequency and improved the quality of the regenerated buds (Liu et al. 2015).

Axillary bud-derived shoots proliferated on MS medium supplemented with 2.22 µM BAP and 0.049 µM IBA (Thepsamran et al. 2007). MS medium containing 1.5 mg/l BAP, 0.5 mg/l Kinetin and 0.1 mg/l IAA led to direct shoot regeneration from nodal explants (Kalimuthu et al. 2007). Shoot regeneration from axillary nodes was observed on MS medium containing BAP (3.0 mg/L) and IBA (1.0 mg/L) along with adenine sulfate (AdS) (25 mg/L), glutamine (50 mg/L), L-arginine (15 mg/L), citric acid (25 mg/l) (Shrivastava and Banerjee 2008). Likewise, axillary shoot bud induction from nodal segments was noticed on MS medium supplemented with BAP 22.2 µM and adenine sulfate 55.6 µM (Datta et al. 2007). MS medium supplemented BAP (6.65 µM) and L-glutamine (25 mg/L) produced an average of 64 buds per aggregate from nodal segments (Mve et al. 2013). Efficient multiple shoot bud induction from the nodal segments was observed on MS medium supplemented with BAP (3.0 mg/L), Kn (1.0 mg /L) and IBA (0.5) mg/L (Anjali et al. 2015).

### *2.4.2 Indirect Regeneration*

*Organogenesis through callus:* Organogenesis is a complex process. Intermediary callus-derived regeneration in *J. curcas* is influenced by several factors such as nature of explant (cotyledons, epicotyls, hypocotyls, leaves, shoot tips, petioles, zygotic embryos), age of explant, media formulations, plant growth regulators (PGRs), genotype, carbohydrate source, gelling agent, and othe conditions like light, temperature and, humidity (Kumar et al. 2015; Mukherjee et al. 2011). Morphogenic callus induction from immature embryos was observed on MS medium supplemented with BAP (1.0 mg/L), IBA (0.5 mg/L), casein hydrolysate (100 mg/L), glutamine (200 mg/L) and $CuSO_4$ (8.0 mg/L); further plant regeneration occurred on MS medium supplemented with BAP (1.0 mg/L), Kn (0.25 mg/L), IBA (0.25 mg/L), polyvinyl pyrrolidone (500 mg/L) and citric acid (30 mg/L) (Varshney and Johnson 2010). Adventitious shoot bud induction without intervening callus formation from leaf explants was observed on MS medium supplemented with 0.90 µM TDZ and 0.98 µM IBA. This study claimed that TDZ treatment was important for better shoot bud induction as compared to BAP (Khurana-kaul et al. 2010). MS medium with BAP (8.0 µM) and IBA (2.0 µM) was effective for both callus mediated organogenesis and subsequent shoot elongation. Addition of adenine sulfate (45 µM), glutamine (15 µM), and proline (10 µM) enhanced the number of multiple shoots per explant (Maharana et al. 2012). MS medium supplemented with 2,4-D (3.0 mg/L) and BAP (1.0 mg/L) induced callus formation and shoot multiplication during developemental stages of seeds

(Rampadarath et al. 2014). Regenerative callus was induced from *in vitro* raised *p*etioles on MS medium augmented with BAP (4.44 μM) and IBA (2.45 μM). Shoot proliferation and elongation were obtained on BAP (2.22 μM) in combination with IAA (8.56 μM) (Singh et al 2014). Embryo culturing was carried out on MS medium with NAA (1.0 mg/L) and BAP (0.5 mg/L) to get higher callus biomass (Hernandez et al. 2015). *In vitro* organogenesis of adventitious shoots of *J. curcas* was observed on MS medium supplemented with purine (2iP) (110.25 μM) in combination with IAA (1.27 μM), and AdS (369.21 μM); shoot development was observed on BAP (4.44 μM), IAA (1.0 μM) and AdS (543 μM). AdS, being an organic additive could interac with cytokinins for induction and elongation of the *in vitro* shoots (Herrera-Cool et al. 2019).

## 2.5 Rationale Behind the Study

Around 30–40% of the total biofuel production in USA and in many countries of Europe and South-East Asia depend on the plant-derived edible oils such as soybean, rapeseed, peanut, sunflower and palm for biodiesel production for blending with petro-fuels. As there is a growing demand and short-supply of the edible oils in Indian subcontinent, the focus has been shifted to the promising tree-borne oilseeds (TBOs) such as Neem (*Azadirachta indica*), Karanj (*Pongamia pinnata*), Mahua (*Madhuca indica*), Jatropha (*Jatropha curcas*) for biodiesel production. This study focuses on *Jatropha curcas* L. or physic nut, a member of the *Euphorbiaceae* family, is a multipurpose deciduous small tree or shrub distributed in many tropical and subtropical regions of Asia including Indian subcontinent and Africa. *J. curcas* draws the attention of many researchers/policy makers/stakeholders for its rapid growth, easy propagation, drought and pest resistance, importantly high seed and oil yield with desired fatty acid composition prerequisites for production of quality biodiesel.

Considerable progress has been made on *J. curcas* in India and other countries during the last few decades as evident from the published reports. However, to realise the full potential of this bioenergy crop, *J. curcas*, we still need extensive survey, selection, collection and evaluation of diverse local and global *Jatropha* germplasm from different eco-geographical locations to check growth parameters, morphology, seed characteristics and yield traits, reaction to pests and diseases followed by assessment of genetic variability and diversity for various desirable attributes. Thorough survey at different ecogeographic zones in India and other countries only can widen the scope of getting quality germplasm for mass multiplication, field trials, performance evaluation and genetic improvement of *Jatropha*.

There was no comprehensive report on *J. curcas* genotypes available from the North-Western regions of our country. An extensive survey was carried out in Punjab, a North-Western state of India for selection of *Jatropha* germplasm from different locations, followed by assessment of the accessions on the basis of genetic variability and divergence in seed traits and oil content. Apart from selection and collection of superior *J. curcas* genotypes, other studies like extraction of seed oil from the *J. curcas* accessions, analysis of oil and FFA content, transesterification are relevant and useful. Development of simple and efficient true-to-type micropropagation protocol through tissue is also an important aspect of research as such techniques help in crop improvement also help in producing quality planting materials for field trials and large scale cultivation. Keeping in view of all these points, the following objectives were framed for the thesis work.

## 2.6 Objectives

- Survey, genetic diversity analyses and selection of elite clones of *Jatropha curcas*
- Analysis of seed oil quality and fatty acid composition
- Micropropagation of elite clones of *Jatropha*

Chapter 3

# Materials & Methods

## 3.1 Materials

### *3.1.1 Chemicals/biochemicals*

Various chemicals, biochemicals and plant tissue culture items were procured from different sources. The chemicals were purchased from Sisco Research Laboratory Pvt. Ltd. Mumbai, Qualigens Fine Chemicals, Merck, CDH Pvt. Ltd., New Delhi, and HiMedia Laboratories Mumbai. All salts and Plant growth regulators (PGRs) were purchased from HiMedia Labs Limited, India and growth hormones from sigma chemicals, USA. For GC analysis, a standard fatty acid methyl ester (FAME) $C_8$–$C_{24}$ was purchased from Sigma-Aldrich. Methanol, potassium hydroxide, hexane and other chemicals were of analytical grade, and purchased from Himedia.

### *3.1.2 Glasswares, Plasticwares and Other Materials*

Different types of glasswares and plasticwares were used, which includes conical flasks (100, 150, 250, 500, and 1000 mL), measuring cylinders (25, 100, 500 and 1000 mL), beakers (250 and 500 mL), culture bottles (8 x 3 inches) and test tubes with plastic caps. All these were brought from Borosil Products Pvt. Ltd. Appendorfs (1.5, 2 mL), tips (200 and 1000 µL) were brought from Tarsons Products Pvt. Ltd. Glass plate, scalpel, forceps, blades, tissue paper, cling film, ethanol, detergent, cotton and others were purchased locally.

## 3.2 Methods

### *3.2.1 Survey and Selection of Candidate Plus Trees (CPTs)*

An extensive survey was carried out to select a total of 31 morphologically superior *Jatropha* CPTs from different locations of Punjab (Fig. 5) having latitude, longitude and altitude ranging 30°20′–31°32′N, 74°18′–76°28′E, and 211−260 m, with average annual rainfall about 700 ± 50 mm respectively (Table 3). 75% rainfall is received during the month of mid-June to September and remaining rainfall from December to February. From each CPT, mature black fruits were collected during December (2013)-January (2014), and black seeds were extracted through the dehulling and shelling process. Healthy and mature seeds from each CPT were dried under the sun for 1-2 days till they attained constant weight and distributed into five lots after cleaning through winnowing and stored in muslin bags under ambient conditions. For each seed lot, out of 100 healthy seeds, ten (10) seeds were chosen randomly to measure length, width, and thickness in millimeters (mm), and their averages were recorded; each measurement was replicated thrice. Likewise, 100-seed weight (expressed in ‘g’) was also measured.

The collected *J. curcas* germplasm in terms of both seed and cuttings was raised and maintained in the nursery located at the Thapar campus to identify elite germplasm. The *Jatropha* seeds from 31 CPTs were submitted to National Bureau of Plant Genetic Resources (NBPGR), New Delhi with the following Indigenous Collection (IC) numbers: 569342 to 569358 (except 569345), 561287 to 561293, 568549 to 568556 (except 568555), and 560678.

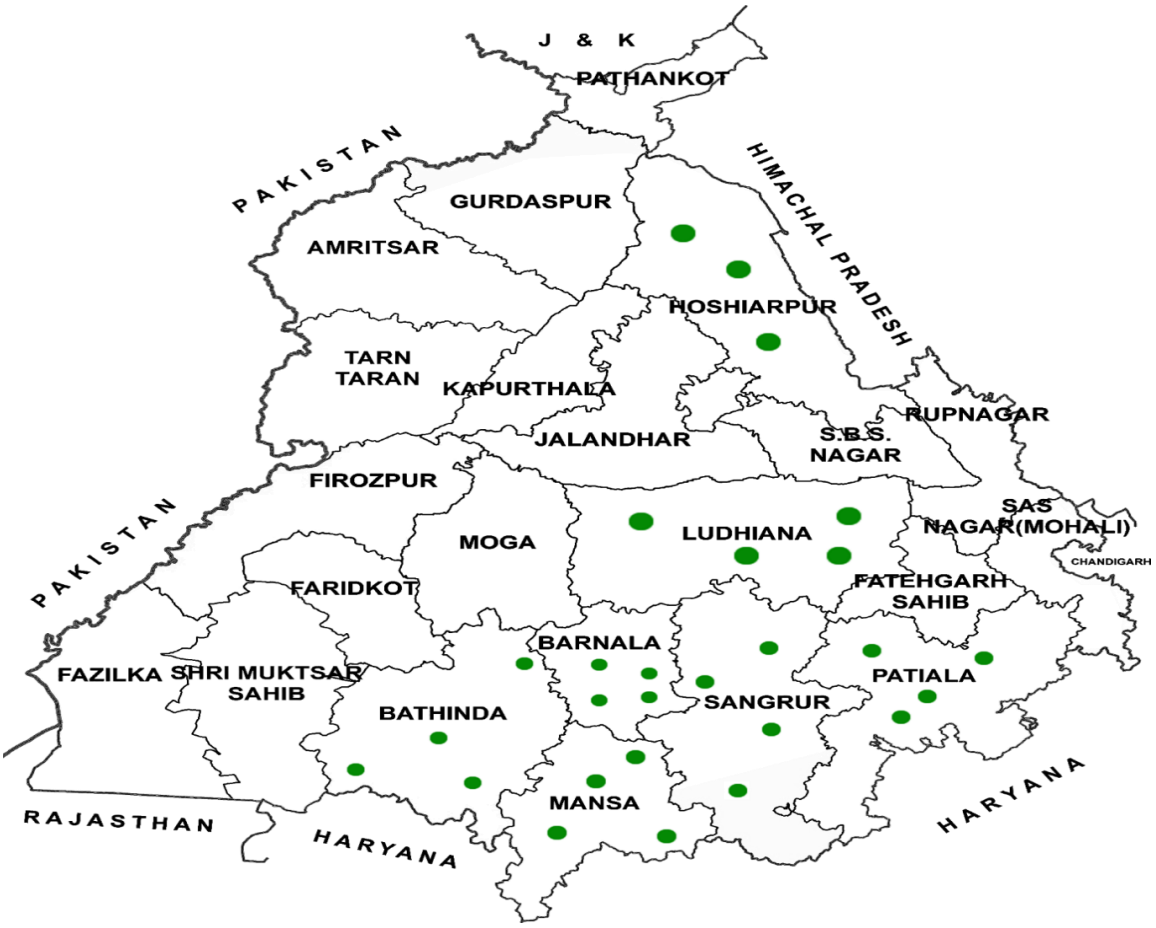


**Fig. 5.** Mapping of the 31 survey sites of *J. curcas* accessions collected from 7 districts of Punjab (Patiala, Barnala, Ludhiana, Sangrur, Mansa, Bathinda and Hoshiarpur)

### *3.2.2 Plant Material and Media Preparation*

On the basis of DBT guidelies as mentioned earlier, the elite genotypes of *J. curcas* were selected and marked as Candidate Plus Trees (CPTs) based on the consistency of seed yield and other desirable morphological attributes. The seeds and cutting raised plants from these CPTs were maintained as germplasm bank at Thapar campus, Patiala. The mother plants were also maintained

in block plantations and watered regularly and supplied with inorganic fertilizers, farmyard manure (FYM) and organic compost. The explants required for *in vitro* tissue culture establishments were taken from these CPTs. Murashige and Skoog's (MS) basal medium was used for all the experiments for this study (Murashige and Skoog 1962).

For the preparation of the 1L MS media, tissue culture bottles were washed and kept overnight in a hot air oven at 70 °C. In 1 L flask, all the major salts, minor salts, and vitamins were initially added with ~300–400 ml of double distilled as per the proportion given in Table 1. The final volume was made-up to 1L with double distilled water. The media were solidified by using 7 gm/L agar and supplied as 3% sucrose as the carbohydrate source. The pH of the medium was set to 5.8 prior to the addition of agar. The required concentrations of plant growth regulators (PGRs) were added to the different media formulations inside laminar air flow chamber as per its solubility mentioned in the Table 2. after melting of the media 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 to check any bacterial or fungal contamination. The *in vitro* cultures were maintained in a plant growth room at 26 ± 2ºC temperature, 55–60% relative humidity (RH), under 12 h photoperiod with a light intensity of 35–40 µmol/$m^2$s spectral flux photon (SFP) of photo-synthetically active 460–700 nm radiations and with 8 hours dark period.

**Table 1.** Composition and Stock Preparation for Murashige and Skoog (MS) basal Medium

**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 | $MgSO_4.7H_2O$ | 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 major salts stock solutions were prepared separately and stored in different bottles.

**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 |

Note: To avoid precipitation, $Na_2MoO_4.2H_2O$ was added first followed by the $H_3BO_4$ during preparation of minor salts stock solutions.

**MS Vitamins:**

| S. No. | Name of vitamins | MS basal conc. (mg/L) | Amount required for 1000X stock (mg/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 were prepared separately in different bottles

| S. No. | Name of chemical | MS basal conc. (mg/L) | Amount required for 1000X stock (mg/L) | Use of stock for 1L medium (mL) |
|---|---|---|---|---|
| 1. | $Fe_2EDTA.2H_2O$ | 30.0 | 30.0 | 1.0 |

Note: MS basal media preparation includes major salts, minor salts, vitamins, $Fe_2EDTA.2H_2O$, 3.0% sucrose, 0.7-0.8% agar. The pH of the media was set at 5.8 using 0.01N HCL or 0.01N NaOH.

**Table 2.** Composition and Stock Preparation for phyohormones

**Various Phytohormones**

| S. No. | Phytohormones | Stock Conc (mg/mL) | Working Conc (mg/L) | Detail of preparation |
|---|---|---|---|---|
| 1. | BAP (6-Benzylaminopurine, Kn (Kinetin) | 2.5 | 2.5 | Both BAP and Kinetin were dissolved in 0.1 N HCl and made the volume by adding sterile distilled water |
| 2. | IBA (Indole butyric acid), IAA (Indole acetic acid) and NOA (β-naphtoxyacetic acid) | 2.0 | 0.1 | Auxins IBA, IAA and NOA were dissolved in 0.1 N KOH, stirred gently and made up the volume by adding sterile distiiled water |
| 3. | TDZ (Thidiazuron) | 2.0 | 0.l | TDZ was dissolved in 0.1 N KOH, stirred gently and made up the volume by adding sterile distiiled water |

### *3.2.3 Processing of Explants and In Vitro Culture Establishment*

The fresh *J. curcas* sprouts of 35–40 cm length were harvested from the mother plants in the month of mid-March to July. The harvested explants were prepared by removing leaves and petiole with only leaving a small portion of petiole attached with the stem. These shoot segments were cut into nodal shoot segments (2.0–3.0 cm) with 1–2 nodes per explant. The nodal shoot explants were initially imbibed in fresh tap water for about 2–3 hours to remove all the adherent dust particles. For surface sterilization, the explants were given the detergent treatment (Tween-20) for about 7–10 minutes with intermittent shakings. The explant containing bottle was then placed under the running water for about 10 minutes to completely remove the detergent solution. The explants were then treated with 5% sodium hypochlorite solution for about 5–7 minutes to overcome bacterial contaminants followed by washing under running tap water for 10 minutes. Finally, the explants were treated with 0.2% bavistin for about 5–7 minutes to kill all the systemic fungal contaminants followed by its washing under running tap water for about 15 minutes. Under the laminar airflow chamber, the nodal explants were given 0.2% mercuric chloride ($HgCl_2$) treatment for 3–5 minutes, followed by washing (4–5 times) with autoclaved double distilled water. Before inoculation of explant material, laminar air flow chamber was sterilized properly followed by heat sterilization of the glass plate, forceps, scalpel and blade. The explants in the form of shoot

tip/nodal segments were carefully placed on the sterile glass plate in the horizontal position. The shoot tip/ nodal segments were given fresh cuts on both the ends. These explants were then cultured on MS medium supplemented with 0.0–5.0 mg/L BAP (6-benzylaminopurine), Kin (Kinetin) and TDZ (thidizuron) separately in glass culture bottles and test tubes. For the optimization of the culture initiation medium and growth of regenerated shoot buds, 0.1–1.0 mg/L IAA (indole-3-acetic acid) and IBA (indole-3-butyric acid) were used along with the combination of 0.5–2.0 mg/L BAP/Kin. After the inoculation of the suitable explants, the cultures were transferred to the plant growth room for establishment.

### *3.2.4 Amplification and Elongation of Shoot Buds*

Multiplication of shoot buds in tissue culture conditions was achieved by repeated transfer and subculture of *in vitro* derived nodal segments along with mother explants. For this purpose, different concentrations and combinations of BAP and IBA were used to check bud breaking from nodal explants along with the evaluation of effects of different concentrations and combinations of BAP 0.25–2.0 mg/L and IBA 0.1–1.0 mg/L on multiplication and growth of newly regenerated shoot buds. In 4 weeks, old culture, newly regenerated shoot buds along with mother explants were repeatedly transferred on MS medium containing BAP 0.25–2.0 mg/L and IBA 0.1–1.0 mg/L for 2-3 transfer. Further, the proliferated shoot buds were separated from each other and sub-cultured on MS medium containing 0.25–0.5 mg/L each BAP and IBA for 2-3 passage for elongation. The elongated shoots were cut into the apical shoot portion and nodal shoot segments. For further induction of multiple shoot buds for the next generation and their subsequent elongation, *in vitro* derived nodal segments were cultured on MS medium supplemented with BAP 0.25–1.0 mg/L and IBA 0.1–1.0 mg/L. The elongated apical shoot portion was used for the root regeneration in the root induction medium.

### *3.2.5 Root Induction*

The *in vitro* elongated shoots were cultured on both half and full-strength MS medium supplemented with 0.0–5.0 mg/L IBA, IAA and NOA (β-naphthoxyacetic acid) separately for root induction.

### *3.2.6 Acclimatization and Transplantation*

The *in vitro* derived rooted plantlets were removed from plant tissue culture bottles and washed thoroughly with autoclaved distilled water to remove adhering basal MS medium and agar to avoid

microbial contamination. These were transplanted on to the sterile autoclaved soil in transparent polybags, these were further covered with inverted polybags to maintain high relative humidity (RH) and incubated under culture room conditions. The plantlets were also sprayed with 1/4$^{th}$ strength MS macro salt solution for proper growth. After 1–2 weeks of acclimatization under *in vitro* conditions, the inverted polybags were made porous to allow a gaseous exchange for the plantlets. The acclimatized plantlets from growth room conditions were shifted to the greenhouse chamber and kept at 28 ± 2 ºC temperature and 60–70% relative humidity for further acclimatization. The plantlets were acclimatized under greenhouse conditions for 8–10 weeks with the gradual removal of inverted polybags and its simultaneous transfer towards 32 ± 2 ºC temperature and 35–40% relative humidity. Finally, the acclimatized plantlets were transferred to larger polybags containing soil, sand, and FYM in the 1:1:1 (v/v) ratio. After 4–5 weeks of plantlet growth under high temperature and low relative humidity, the acclimatized plantlets were shifted to polybags and pots under nursery conditions. Various media formulatrions were adopted for micropropagation of *J cucas* L. A schematic view is shown in Fig. 6.

### *3.2.7 Experiment design*

All the experiments were set up in a completely randomized design (CRD) and repeated thrice with a minimum of 10 replicates per treatment and explant was cultured per test tube/glass bottle. The *in vitro* raised plantlets were regularly sub-cultured on to fresh medium after a regular interval of 4–5 wk. Data recording on culture response, number of shoots and roots, length of shoot and root were carried out at regular intervals.

### *3.2.8 Extraction of Seed Oil and Analysis of FFA Content*

*J. curcas* seed oil % on kernel basis was determined using a Soxhlet apparatus (Kumar and Singh 2014). Briefly, 100 g seeds from each CPT were sun-dried for 1–2 days, followed by drying in a hot air oven at 40°C till they attain constant weight and then seeds were broken to extract the kernels. For each seed sample, 50 g kernel was broken into small pieces using mortar and pestle and kept in the middle chamber of the Soxhlet apparatus for oil extraction using hexane as a solvent. The extraction process was carried out at 70–80°C for 8–10 hrs. During this process, hexane containing the extracted *Jatropha* oil was collected in a round bottom flask for boiling, which allowed hexane to get evaporated but not the oil as the boiling point of the latter was more than hexane. To remove hexane completely from seed oil, the flask was kept in a vacuum rotary

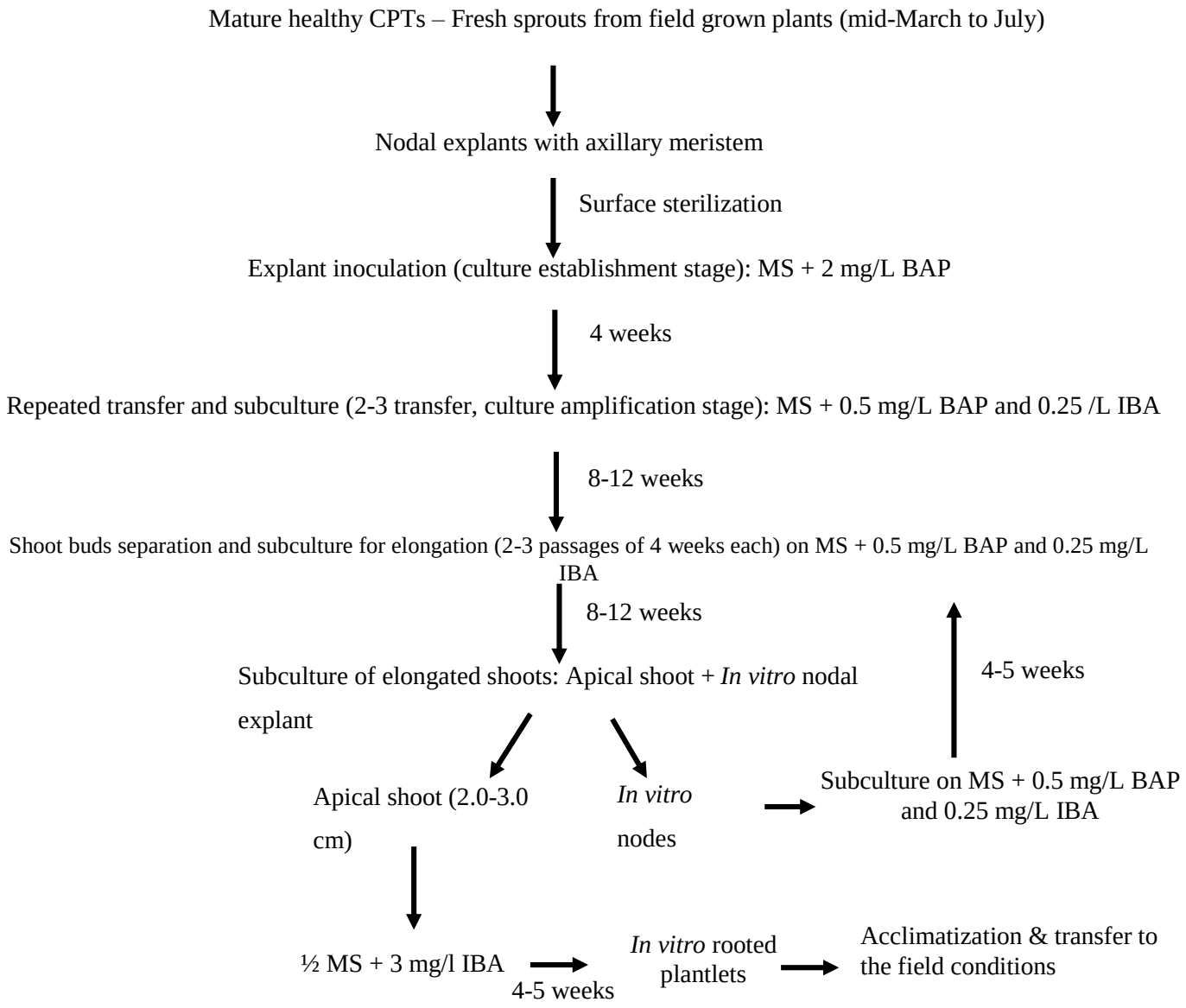


**Fig. 6.** A schematic view of media formulations for micropropagation of *J. curcas* L.

evaporator. The oil % was calculated using the following equation

$$\% \text{ Oil} = [(W_b – W_a) \times 100]/\text{kernel weight} \quad [1]$$

where $W_a$ was the weight of the empty flask and $W_b$ refers to the weight of flask containing the extracted oil. The free fatty acid (FFA) content of each seed oil sample was determined by the titrimetric method (Rukunudin et al. 1998). Briefly, 10 g of oil was dissolved in a mixture of ethanol and diethyl ether (1:1 volume ratio) and titrated with 0.1 M KOH solution using phenolphthalein as an indicator. FFA concentration in seed oil was calculated as percentage oleic acid based on the following equation

$$\% \text{ FFA as Oleic acid} = [\text{alkali volume (mL)} \times \text{alkali normality} \times 28.2]/\text{Sample weight (g)} \quad [2]$$

### *3.2.9 Transesterification*

The extracted *J. curcas* seed oil samples were treated with methanol in presence of KOH to produce fatty acid methyl esters. Briefly, 10 g of each seed oil was mixed with 2.2 mL methanol (1:6 oil-to-alcohol mole ratio) and 0.1 g KOH (1% w/w of oil) in 50 ml beaker. The reaction mixture was kept on hot air magnetic stirrer at 70°C for 2 h with continuous stirring using a magnetic bead. The mixture was then shifted to a separating funnel and kept undisturbed overnight to allow complete separation of two layers in the form of methyl esters and glycerol. After removal of glycerol, the remaining product was washed 2–3 times with an aqueous solution of HCl (0.5 wt %) and NaCl (5.0 wt %) and dried over anhydrous magnesium sulphate, and residual methanol was removed in a rotary evaporator at 70°C (Ghesti et al. 2007). Proton nuclear magnetic resonance ($^1$H NMR) method was employed to assess the quality, particularly with regard to FFA content, of the biodiesel i.e., fatty acid methyl ester as-synthesized (Satyarthi et al. 2009). For $^1$H NMR, 15–20 mg of the sample was dissolved in 0.6 mL of $CDCl_3$; the spectrum was recorded at 298 K on a Jeol ECS 400 MHz spectrometer (during the measurement, 64 scans were taken for each sample). A standard 5 mm quadronuclei ($^1$H, $^{31}$P, $^{13}$C, and $^{15}$N) probe (QNP) was used. The other related parameters such as acquisition time of 3.9 s, relaxation delay of 1 s, flip angle of 45°, and a sweep width of 4.139 kHz were employed during the spectral measurements.

### *3.2.10 Fatty acid composition by gas chromatography*

The individual fatty acid methyl esters were used for quantitative analysis of fatty acid composition by gas chromatography (GC). An Agilent EC-5GC instrument equipped with flame ionization detector, automatic sample injector along with a separation column namely SP-2560 (30.0 m x 0.53 mm x 1.2 µm) was used. The other conditions were: inlet temperature (150ºC), detector temperature (260ºC), split ratio (19:1), oven temperature programme (140ºC to 240ºC at 4ºC/min), hold for 15 min, injection volume (1.0 µL); carrier gas: $N_2$ at 2 mL/min; air flow: 450 mL/min; $H_2$ flow: 45 mL/min. Under these conditions, GC chromatograms of the standards (procured from Sigma-Aldrich, USA) and samples were obtained with an injection volume of 1.0 µL. The chromatographic run time for each sample was around 60 min.

### *3.2.11 Statistical analysis*

The descriptive statistical data such as mean, standard deviation (SD), variance and range were generated on the basis of fatty acid composition of the seed oil samples from the *J. curcas* accessions. SPSS software (Statistical Product and Service Solutions) Version 17 (IBM, New

York, USA) was employed in statistical analysis. Analysis of variance (ANOVA) was carried out using the CPCS1 statistical software developed by Punjab Agricultural University, Punjab, India (Cheema and Singh 1990). The variability, broad sense heritability, genetic advance as percentage of mean, genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), and linear correlation coefficients for the traits under study were calculated using the methods as suggested by different researchers (Burton 1952; Johnson et al. 1955; Sendecor and Cochran 1967). Non-hierarchical (K-means) Euclidean cluster analysis was carried out using IBM SPSS Statistics 21 software to assess the extent of broad genetic divergence (Sachan et al. 2004).

Chapter 4

# Results & Discussion

## Objective-1: Survey, genetic diversity analyses and selection of elite clones of *Jatropha curcas*

*J. curcas* draws the attention of many researchers for their rapid growth, easy propagation, drought tolerance, pest resistance, and, most importantly, high seed yield and oil content, which are prerequisites for the biodiesel production. In order to search superior genotypes, a thorough and extensive survey was carried out in the different regions of the North-Western part of India (the state of Punjab) for selection of *Jatropha* germplasm; assessment of the accessions was done on the basis of genetic variability and divergence study in seeds traits and oil content. A total of 31 CPTs were selected based on the desirable traits like height, crown spread, stem girth, disease resistance, branching pattern, fruits per cluster, seed yield of the individual *J. curcas* plantations. Experimental details are elaborated in the following sections.

## 4.1Results and Discussion (Objective-1)

### *4.1.1 Survey and Selection of Candidate Plus Trees (CPTs)*

The state of Punjab is a part of the Indo-Gangetic alluvial plane, composed of sediments of the Siwalik Hills and the Himalayas, having the following physiographic units: Shiwalik hills, Piedmont plain, Alluvial plain, Sand plain, Flood plain and Palaeochannels; which can be further classified into a number of varying agro-climatic zones based on homogeneity, rainfall pattern, soil texture, and cropping patterns. A thorough survey was carried out to select a total of 31 morphologically superior *Jatropha* CPTs from different locations of Punjab having latitude, longitude and altitude ranging 30°20′-31°32′N, 74°18′-76°28′E, and 211−260 m, with average annual rainfall about 700 ± 50 mm respectively (Table 3). The CPTs were selected based on salient morphological attributes of the individual *J. curcas* plantations, like plant height, seed yield per plant (around 2 kg), no. of fruits per cluster, branching pattern, crown spread, stem girth, disease resistance (Mishra, 2009). The *Jatropha* seeds from 31 CPTs were submitted to National Bureau of Plant Genetic Resources (NBPGR), New Delhi with the following Indigenous Collection (IC) numbers: 569342 to 569358 (except 569345), 561287 to 561293, 568549 to 568556 (except 568555), and 560678 (Table 4). The collected *J. curcas* germplasm in terms of both seed and cuttings was raised and maintained in the nursery located at the Thapar campus to identify elite germplasm.

### *4.1.2 Variability in Seeds*

The seeds from the *J. curcas* CPTs showed considerable variation about the shape, size, and color of the seed coats (Fig. 7). As evident from ANOVA, significant differences ($P<0.05$) were noticed in the seed characteristics between the accessions (Table 4). TJS-46#04, TJS-27#108 and TJS-06#24 were found to be comparable in terms of maximum seed length (i.e., 18.49 mm, 18.48 mm and 18.47 mm, respectively). Minimum seed length was noticed in TJS-15#11 (16.18 mm) followed by TJS-02#01 (16.60 mm). Seed width ranged from 10.65 mm to 11.82 mm. TJS-33#14 showed maximum seed width (11.82 mm) followed by TJS-46#04 (11.69 mm); minimum seed width was noticed in TJS-42#01 (10.65 mm). The seed thickness varied from TJS-19#01 (7.89 mm) to TJS-39#73 (8.85 mm).

### *4.1.3 Seed Weight and Oil Content*

The CPTs showed significant variation in terms of both 100-seed weight and oil content (Table 4). The range of 100-seed weight varied markedly from 35.10 g (TJS-02#01) to 77.34 g (TJS-31#13). For the majority of the accessions, the values were found to be in the range of 60-70 g; however, there were 6 accessions that showed the values above this range. With regard to seed oil content, variation was also significant between the accessions. For this trait, the values were between 13.74% (TJS-02#01) and 54.37% (TJS-04#42). 24 accessions showed 30-40% oil content; 3 accessions showed more than 40% oil content, and for the remaining, it was less than 30%.

**Table 3:** Ecogeographical characteristics of the survey areas in Punjab for collection of *J. curcas* germplasm

| District | Location | | | Agro-climatic zones of Punjab |
|---|---|---|---|---|
| | Altitude | Latitude | Longitude | |
| Patiala | 259 m | 30°-20' N | 76°-28' E | Central plain zone |
| Sangrur | 259 m | 30°-20' N | 76°-28' E | Central plain zone |
| Ludhiana | 259 m | 30°-20' N | 76°-28' E | Central plain zone |
| Barnala | 259 m | 30°-20' N | 76°-28' E | Central plain zone |
| Mansa | 211m | 30°-58' N | 74°-18' E | Western zone |
| Bathinda | 211 m | 30°-58' N | 74°-18' E | Western zone |
| Hoshiarpur | 260 m | 31°-32' N | 75°-55' E | Sub-mountain undulating zone |

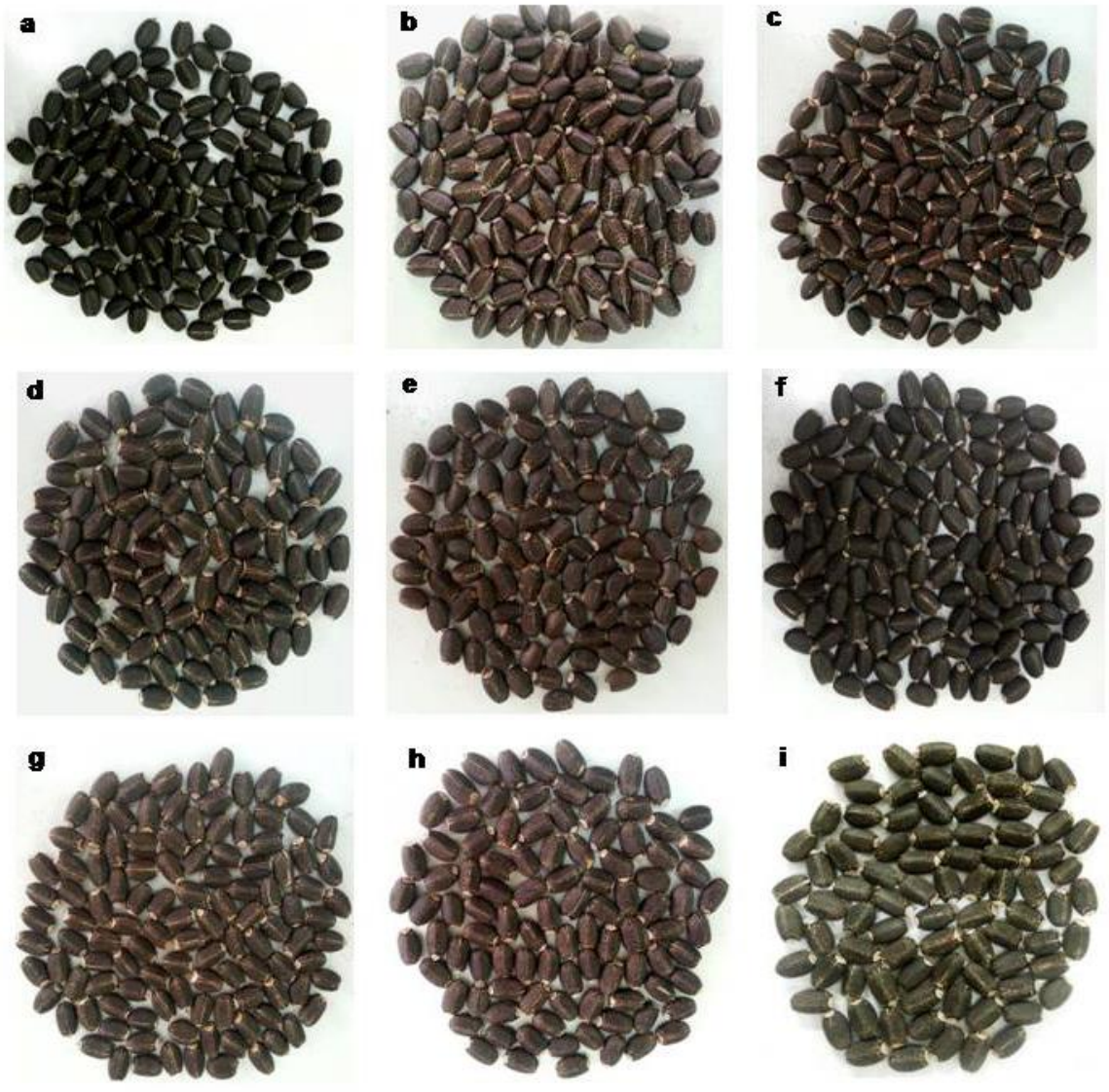


**Fig. 7.** Harvested mature seeds from different *J. curcas* accessions: TJS-01#04 (a); TJS-04#42 (b); TJS-05#10 (c); TJS-07#05 (d); TJS-15#11 (e); TJS-23#17 (f); TJS-27#108 (g); TJS-29#07 (h); TJS-36#23 (i)

**Table-4:** Seed traits and oil content variability in *J. curcas* accessions collected in the state of Punjab, India

| CPT | Acc. No. | NBPGR IC No. | Seed length (mm) | Seed width (mm) | Seed thickness (mm) | 100-seed weight (g) | Oil content (%) |
|---|---|---|---|---|---|---|---|
| 1 | TJS–01#04 | IC-568549 | 17.14 | 10.69 | 8.07 | 65.58 | 37.35 |
| 2 | TJS–02#01 | IC-569343 | 16.60 | 10.66 | 8.17 | 35.10 | 13.74 |
| 3 | TJS–04#42 | IC-561287 | 17.23 | 11.28 | 8.39 | 69.86 | 54.37 |
| 4 | TJS–05#10 | IC-561288 | 17.04 | 11.05 | 7.89 | 66.65 | 42.05 |
| 5 | TJS–06#24 | IC-561289 | 18.47 | 11.56 | 8.45 | 69.95 | 33.16 |
| 6 | TJS–07#05 | IC-569342 | 17.92 | 10.69 | 8.55 | 74.07 | 29.85 |
| 7 | TJS–15#11 | IC-569344 | 16.18 | 10.97 | 8.07 | 43.36 | 18.24 |
| 8 | TJS–17#01 | IC-561290 | 17.73 | 10.88 | 8.48 | 63.73 | 32.59 |
| 9 | TJS–19#01 | IC-568551 | 17.48 | 10.78 | 7.89 | 65.73 | 38.41 |
| 10 | TJS–21#01 | IC-568550 | 17.41 | 11.14 | 8.59 | 60.26 | 33.58 |
| 11 | TJS–23#17 | IC-569346 | 18.29 | 11.48 | 8.22 | 66.50 | 45.17 |
| 12 | TJS–25#01 | IC-568552 | 17.24 | 11.10 | 8.47 | 60.65 | 38.86 |
| 13 | TJS–26#39 | IC-561292 | 17.87 | 11.01 | 8.37 | 60.90 | 35.65 |
| 14 | TJS–27#108 | IC-568554 | 18.48 | 11.21 | 8.44 | 69.58 | 32.93 |
| 15 | TJS–28#01 | IC-568556 | 17.32 | 11.28 | 8.18 | 67.86 | 35.52 |
| 16 | TJS–29#07 | IC-569347 | 17.81 | 11.51 | 8.15 | 73.99 | 35.44 |
| 17 | TJS–30#06 | IC-561291 | 17.66 | 11.42 | 8.54 | 71.65 | 35.45 |
| 18 | TJS–31#13 | IC-569348 | 17.96 | 10.91 | 8.36 | 77.34 | 36.04 |
| 19 | TJS–32#16 | IC-569349 | 17.92 | 11.38 | 8.36 | 68.35 | 35.51 |
| 20 | TJS–33#14 | IC-569350 | 17.45 | 11.82 | 8.04 | 62.94 | 33.28 |
| 21 | TJS–34#37 | IC-569351 | 17.20 | 11.06 | 8.67 | 59.55 | 34.98 |
| 22 | TJS–35#01 | IC-569352 | 17.11 | 11.31 | 8.55 | 58.06 | 35.20 |
| 23 | TJS–36#23 | IC-561293 | 17.17 | 11.27 | 8.50 | 71.33 | 36.12 |
| 24 | TJS–37#33 | IC-560678 | 17.91 | 11.38 | 8.72 | 69.01 | 33.95 |
| 25 | TJS–38#03 | IC-568553 | 17.72 | 11.42 | 8.52 | 62.59 | 36.54 |
| 26 | TJS–39#73 | IC-569353 | 17.30 | 11.42 | 8.85 | 70.61 | 28.56 |
| 27 | TJS–40#05 | IC-569354 | 18.35 | 11.38 | 8.70 | 64.17 | 36.94 |
| 28 | TJS–41#14 | IC-569355 | 17.66 | 11.30 | 8.73 | 68.23 | 31.54 |
| 29 | TJS–42#01 | IC-569356 | 16.74 | 10.65 | 8.15 | 50.33 | 39.64 |
| 30 | TJS–46#04 | IC-569357 | 18.49 | 11.69 | 8.77 | 60.79 | 39.58 |
| 31 | TJS–49#01 | IC-569358 | 17.65 | 11.22 | 8.61 | 64.70 | 32.20 |
| SEm(±) | | | 0.11 | 0.51 | 0.62 | 0.17 | 1.54 |
| | CD at 5% | 0.41 | 0.28 | 0.31 | 0.67 | 2.02 | |

### *4.1.4 Genetic Variability Studies in Seed Traits*

The extent of genetic variability expressed in different seed and oil traits was examined by the studies that included genotypic and phenotypic variance, GCV, PCV along with broad-sense

heritability and genetic advance. The magnitude of variability corresponding to some seed traits of *J. curcas* are shown in Table 5. For the traits namely length, width and thickness, the magnitude of both phenotypic variance and PCV were found to be higher in comparison to the corresponding genotypic variance and GCV. The extent of both genotypic and phenotypic variance was highest and comparable for the 100-seed weight (i.e., around 76) followed by nearly comparable for seed oil content (i.e., around 49). A similar pattern was noticed for GCV and PCV; the values for oil content was highest and comparable (i.e., around 20) followed by 100-seed weight (i.e., around 14). The highest broad-sense heritability of 99% was recorded for 100-seed weight followed by oil content (97%) and seed length (81%); whereas seed thickness showed the lowest heritability of 17% followed by 32% as found in seed width. The genetic advance as represented by percent of mean ranged from 0.30% for seed thickness to 17.83% for 100-seed weight. The correlation coefficient between seed and oil traits are presented in Table 6. A positive and significant correlation was observed between 100-seed weight and oil content (r = 0.517). Likewise, seed length and 100-seed weight were positively and significantly correlated. Seed length showed a positive correlation with oil content and seed width. Seed width showed a positive correlation with all other traits under study. Seed thickness was positively correlated with other traits except for oil content as it showed a negative and insignificant correlation.

**Table 5:** Determination of genotypic and phenotypic variables for seed and oil traits in *J. curcas*

| Traits | Variance | | Coefficient of variation (%) | | Heritability (broad sense) | Genetic advance as % of mean |
|---|---|---|---|---|---|---|
| | Genotypic | Phenotypic | Genotypic | Phenotypic | | |
| Seed length | 0.48 | 0.59 | 3.93 | 4.38 | 81 | 1.28 |
| Seed width | 0.24 | 0.75 | 4.39 | 7.70 | 32 | 0.57 |
| Seed thickness | 0.13 | 0.75 | 4.28 | 10.36 | 17 | 0.30 |
| 100-seed weight | 75.91 | 76.08 | 13.55 | 13.56 | 99 | 17.83 |
| Oil content | 47.91 | 49.45 | 19.82 | 20.13 | 97 | 14.04 |

### *4.1.5 Divergence Analysis*

Based on the non-hierarchical *K*-Means cluster analysis, the CPTs were grouped into 6 clusters on the basis of seed and oil traits (Table 7). 12 accessions were included in cluster VI followed by cluster I (9 accessions) and cluster IV (6 accessions) and cluster II (2 accessions), whereas both the clusters III and V included only 1 accession. The inter-cluster distances are presented in Table 8. The highest inter-cluster distance i.e., 49.114 was found between the clusters II and III followed

by the value 36.389 between the clusters II and VI. The minimum inter-cluster distance i.e., 6.194 was noted between cluster I and IV. Cluster mean values for seed and oil traits are shown in Table 9.

**Table 6:** Phenotypic correlation coefficient between seed and oil traits in *J. curcas.*

| Traits | 100-seed weight | Oil content | Seed length | Seed width |
|---|---|---|---|---|
| Oil content | 0.517 | | | |
| Seed length | 0.620 | 0.312 | | |
| Seed width | 0.336 | 0.246 | 0.479 | |
| Seed thickness | 0.226 | − 0.037 | 0.402 | 0.337 |

Correlation is significant at 5% level.

**Table 7:** Non-hierarchical K-Means clusters for seed and oil traits in *J. curcas* accessions

| Cluster | Number of Accessions | Accession names * |
|---|---|---|
| I | 9 | TJS-17#01 (2.725), TJS-21#01 (1.903), TJS-26#39 (1.519), TJS-33#14 (1.819), TJS-34#37 (2.415), TJS-35#01 (3.905), TJS-38#03 (2.122), TJS-40#05 (3.401), TJS-49#01 (3.675) |
| II | 2 | TJS-02#01 (4.711), TJS-15#11 (4.711) |
| III | 1 | TJS-04#42 (0.000) |
| IV | 6 | TJS-01#04 (3.220), TJS-05#10 (3.029), TJS-19#01 (2.363), TJS-23#17 (5.448), TJS-25#01 (3.943), TJS-46#04 (3.775) |
| V | 1 | TJS-42#01 (0.000) |
| VI | 12 | TJS-06#24 (1.376), TJS-07#05 (4.941), TJS-27#108 (1.741), TJS-28#01 (3.685), TJS-29#07 (3.499), TJS-30#06 (1.905), TJS-31#13 (6.783), TJS-32#16 (3.229), TJS-36#23 (2.549), TJS-37#33 (2.026), TJS-39#73 (5.166), TJS-41#14 (3.505) |

*Euclidean distance for each cluster member is indicated within the parenthesis.

**Table 8:** Inter-cluster distances based on seed and oil traits in *J. curcas* accessions

| Cluster | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| I | – | 29.313 | 21.370 | 6.194 | 12.668 | 9.164 |
| II | | – | 49.114 | 34.912 | 26.128 | 36.389 |
| III | | | – | 15.188 | 24.476 | 20.737 |
| IV | | | | – | 14.035 | 9.373 |
| V | | | | | – | 21.550 |
| VI | | | | | | – |

**Table 9:** Cluster mean value for seed and oil traits in *J. curcas* accessions

| Cluster | Seed Length | Seed Breadth | Seed Thickness | Seed Weight | Oil Content |
|---|---|---|---|---|---|
| I | 17.61 | 11.25 | 8.50 | 61.88 | 34.55 |
| II | 16.39 | 10.82 | 8.12 | 39.23 | 15.99 |
| III | 17.23 | 11.28 | 8.39 | 69.86 | 54.37 |
| IV | 17.61 | 11.13 | 8.22 | 64.32 | 40.24 |
| V | 16.74 | 10.65 | 8.15 | 50.33 | 39.64 |
| VI | 17.80 | 11.28 | 8.49 | 71.00 | 33.67 |

### *4.1.6 Discussion*

Currently, large-scale cultivation of superior *J. curcas* varieties is being promoted in many countries because they can serve as potential renewable substitute for fossil fuels. Seed yield and oil content are regarded as two major traits for any biodiesel crop. To realize the full potential of this biodiesel crop, we still need extensive survey, selection and collection of both local and global *Jatropha* germplasm from different eco-geographic locations followed by assessment of genetic variability and diversity for various desirable traits.

Apart from annual seed yield per plant, an important attribute of the elite *Jatropha* accessions includes 30–40% oil content with the presence of high amounts of mono-unsaturated fatty acids

such as C16:1 and C18:1 (Knothe, 2009). Seed oil content is one of the important parameters that need to be considered during selection of a candidate plus trees for any TBO. Seed stocks of most *J. curcas* accessions under study were acceptable in terms of their contribution to overall yield of raw vegetable oil by the extraction process as described earlier. Kaushik and Bhardwaj (2013) collected *J. curcas* seeds from 14 different geographical locations in India; the oil content was found to vary significantly i.e., 11.68% to 42.08% depending on location and soil type. During survey, the salient morphological attributes of the individual *J. curcas* plantations were first recorded; the CPTs were selected on the basis of some desirable traits such as seed yield (2–5 kg/plant), number of mature fruits per raceme, branching patterns with compact canopy, height, crown spread, collar diameter, disease resistance, plant hardiness, and synchronized maturity. Although each of these attributes is important, some criteria are considered preferentially over others during systematic evaluation and selection of a CPT (Mishra, 2009).

Germplasm in the form of seeds for any tree species are known to vary considerably with regard to morphological and physiological parameters depending on the prevailing eco-geographic conditions. Such variations are due to both genotype as well as their adaptation to different environmental conditions (Mathur et al., 1984; Senger et al., 2016). Due to wide distribution range of *J. curcas*, there is a scope to identify genetic variation in the germplasm. The CPTs under study are adaptive to different agro-climatic zones of Punjab that include wide range of rainfall, temperature and soil types. The analyses of seed and oil traits of the mature seeds revealed considerable variation between the CPTs. The extent of variability was analyzed by some useful parameters such as genotypic and phenotypic variances (GV and PV) along with genotypic and phenotypic coefficient of variation (GCV and PCV). As revealed in the earlier reports, the GCV was found to be lower than PCV for all the seed and oil traits namely 100-seed weight, seed:kernel (S/K) ratio, oil content, seed length, seed width and seed thickness which suggests that the characteristics were least influenced by the environment (Ginwal et al., 2004; Kaushik et al., 2007; Gohil and Pandya, 2008).

Heritability is an important aspect in the areas of conventional breeding and genetics as it provides an estimate of the role of genetic factors that influence variation in the phenotypic traits. Nearly 100% heritability was noticed for number of fruits per plant, and it was 88.79% for number of inflorescence per plant in *J. curcas* (Mohapatra and Panda, 2010). Likewise, high heritability and genetic advance were reported for the following traits: fruit yield per plant ($H2$ = 92.22 and GA =

76.82) and seed yield per plant ($H2$ = 88.67 and GA = 56.66) (Tripathi et al., 2013). Heritability values with genetic advance were duly considered for screening and selection of the CPTs under study. High broad sense heritability with moderate genetic advance was observed in the cases of 100-seed weight ($H2$ = 99.00 and GA = 17.83) and oil content ($H2$ = 97.00 and GA = 14.04). These traits need to be considered for direct selection of the elite *J. curcas* germplasm. Seed length ($H2$ = 81.00 and GA = 1.28) showed high heritability but with lower genetic advance. This could be due to the presence of non-additive gene effects and higher genotypic and environmental interactions. The traits like seed width and seed thickness recorded low heritability and low genetic advance indicating the predominance of non-additive gene interactions. As reported earlier, significant positive correlation was found between seed oil content and 100-seed weight suggesting the effectiveness of indirect selection on the basis of these traits (Rao et al., 2008; Tripathi et al., 2013). However, there was little positive correlation between 100-seed weight and oil content (r = 0.235) (Halilu et al., 2011). In this study, positive and significant correlation was noticed between the traits like 100-seed weight and oil content (r = 0.517). The trait like seed length was found to be positively correlated with 100-seed weight, oil content, seed thickness and seed width. The traits showing considerable correlation are likely to be controlled by the genes which are closely linked.

As evident from the *K*-Means cluster analysis, there was no direct correlation between agro-climatic diversity and genetic diversity. Wider genetic diversity was noticed between the clusters II and III followed by the clusters II and VI. Significant variation was noticed between the clusters with regard to oil content and seed weight. Cluster III with a single member showed maximum oil content and higher values for the seed traits like seed weight, length, breadth and thickness. In addition to Cluster III, both the clusters IV and VI showed considerably higher values for the oil content and the other seed traits. Particularly, the CPTs of the clusters III, IV and VI could be used for selection and further tree improvement programmes.

### 4.1.7 Conclusions

Extensive survey and analysis of the seed and oil traits helped to identify a number of superior *J. curcas* genotypes which could be used for large-scale plantations with proper agronomic practices. The *J. curcas* accessions namely TJS–04#42, TJS–05#10, TJS–19#01, TJS–23#17, TJS–25#01, TJS–42#01, TJS–46#04 were quite promising in terms of both 100-seed weight and

importantly more than 35% oil content (Table 4). All these *J. curcas* CPTs of this study are quite promising and can serve the purpose of germplasm exchange, mass propagation, evaluation of their field performance through multi-location trials, micropropagation for generation of true-to-type propagules, genetic improvement through conventional/marker-assisted breeding, somaclonal variants, mutant population, doubled haploids, inter-specific hybridization and transgenics for the desired phenotypic traits such as increasing seed yield and oil content per unit area on a sustainable basis. Apart from specific breeding purposes, the selected *J. curcas* genotypes showing more than 30% oil content will be useful for cultivation to improve seed yield, and to maximize oil extraction process for both fatty acid profile analysis and biodiesel production.

## Objective-2: Analysis of seed oil quality and fatty acid composition

*Jatropha curcas* L. is recognized as one of the important non-edible tree borne oilseeds (TBOs) in India and many other tropical countries. *J. curcas* is a promising bioenergy crop as its seed oil is a suitable feedstock for biodiesel production. This section focused on a study of 19 *J. curcas* accessions for seed oil extraction, analysis of FFA content, transesterification, quality checking of the fatty acid methyl esters by $^{1}$H NMR followed by FAC analysis by gas chromatography. As revealed by gas chromatography the contents of the four major fatty acids (palmitic acid, stearic acid, oleic acid and linoleic acid) showed variation in the different seed oils. *The J. curcas* accessions could be distinctly grouped into two categories based on oleate-rich and linoleate rich seed oils. All the details are elaborated in the following section.

## 4.2 Results and Discussion (Objective-2)

### *4.2.1 FFA Content Analysis*

Free Fatty Acid (FFA) content and Fatty Acid Composition (FAC) of the *J. curcas* accessions were analysed as shown in Table 10. FFA content was determined by titrimetric method and the values ranged from 0.21–1.82% in most of the oil samples except one accession i.e., TJS-02#01 (4.81%). 14 out of 19 oil samples of the study showed less than 1.0% FFA content which is consistent with the earlier reports. Aminul Islam et al. (2012) analyzed *J. curcas* seed oil from 24 candidate plus plants (CPPs) of 9 geographical origins. The values of FFA content ranged from 0.3–2.3 %, and it was less than 1% for most of the CPPs.

**Table 10:** Oil content (%, w/w), FFA content (%, w/w), and fatty acid composition (%, w/w) of seed kernels from the selected *J. curcas* accessions

| S. No. | TU Acc. No. | NBPGR IC No. | Oil content | FFA content | Palmitic acid | Stearic acid | Oleic acid | Linoleic acid | Total UFA |
|---|---|---|---|---|---|---|---|---|---|
| 1. | TJS-01# 04 | IC-568549 | 37.35 | 1.22 | 12.44 | 7.94 | 46.36 | 28.72 | 75.08 |
| 2. | TJS-02#01 | IC-569343 | 13.74 | 4.81 | 12.65 | 6.38 | 33.22 | 45.90 | 79.12 |
| 3. | TJS-04#42 | IC-561287 | 54.37 | 0.48 | 14.44 | 5.66 | 38.36 | 40.54 | 78.90 |
| 4. | TJS-05#10 | IC-561288 | 42.05 | 0.99 | 17.05 | 4.39 | 30.57 | 46.25 | 76.82 |
| 5. | TJS-06#24 | IC-561289 | 33.16 | 0.34 | 11.49 | 6.53 | 41.57 | 39.89 | 81.46 |
| 6. | TJS-07#05 | IC-569342 | 29.85 | 0.24 | 11.03 | 5.44 | 38.52 | 44.26 | 82.78 |
| 7. | TJS-15#11 | IC-569344 | 18.24 | 1.82 | 10.77 | 6.06 | 42.84 | 40.98 | 83.82 |
| 8. | TJS-17#01 | IC-561290 | 32.59 | 0.93 | 13.28 | 5.12 | 41.93 | 38.68 | 80.01 |
| 9. | TJS-19#01 | IC-568351 | 38.41 | 1.04 | 10.71 | 6.87 | 44.17 | 36.68 | 80.85 |
| 10. | TJS-21#01 | IC-568550 | 33.58 | 0.40 | 11.74 | 5.68 | 32.16 | 48.95 | 81.11 |
| 11. | TJS-23#17 | IC-569346 | 45.17 | 0.62 | 15.70 | 6.00 | 40.02 | 35.28 | 75.30 |
| 12. | TJS-25#01 | IC-568552 | 38.86 | 0.87 | 10.39 | 5.80 | 45.86 | 34.84 | 80.70 |
| 13. | TJS-27#108 | IC-568554 | 32.93 | 1.00 | 10.72 | 5.86 | 42.25 | 40.50 | 82.75 |
| 14. | TJS-28#01 | IC-568556 | 35.52 | 0.37 | 11.96 | 4.34 | 32.60 | 50.02 | 82.62 |
| 15. | TJS-29#07 | IC-569347 | 35.44 | 0.78 | 12.78 | 6.08 | 38.60 | 41.38 | 79.98 |
| 16. | TJS-30#06 | IC-561291 | 35.45 | 0.37 | 13.21 | 4.36 | 38.98 | 42.02 | 81.00 |
| 17. | TJS-31#13 | IC-569348 | 36.04 | 0.33 | 8.64 | 5.89 | 44.18 | 40.72 | 84.90 |
| 18. | TJS-32#16 | IC-569349 | 35.51 | 0.22 | 11.94 | 5.60 | 32.20 | 48.50 | 80.70 |
| 19. | TJS-33#14 | IC-569350 | 33.28 | 0.21 | 14.35 | 4.79 | 26.26 | 53.78 | 80.04 |

*FFA* free fatty acid; *UFA* unsaturated fatty acid
Values are the means of triplicate assay.

### *4.2.2 Transesterification and FAC Analysis*

The individual seed oil samples were used to carry out transesterification for biodiesel production. The quality of the resulting fatty acid methyl esters (FAME) was assessed by $^{1}$H NMR analysis. This technique is also known to be useful for detection of the presence FFA in the oil samples (Satyarthi et al., 2009). The distinct peak of the biodiesel (δ value ~3.67) corresponding to the *J. curcas* accession TJS-01#04 seed oil is evident in the $^{1}$H NMR spectra (Fig. 8). The data indicate that conversion of triglycerides to methyl esters occurred efficiently. Similar results were also obtained for the remaining accessions (data not shown). Keeping in view, the FAME samples as prepared in the study were used in FAC analyses by gas chromatography (Fig. 9), and the details are shown in Table 10. The descriptive statistical data are presented in Table 11.

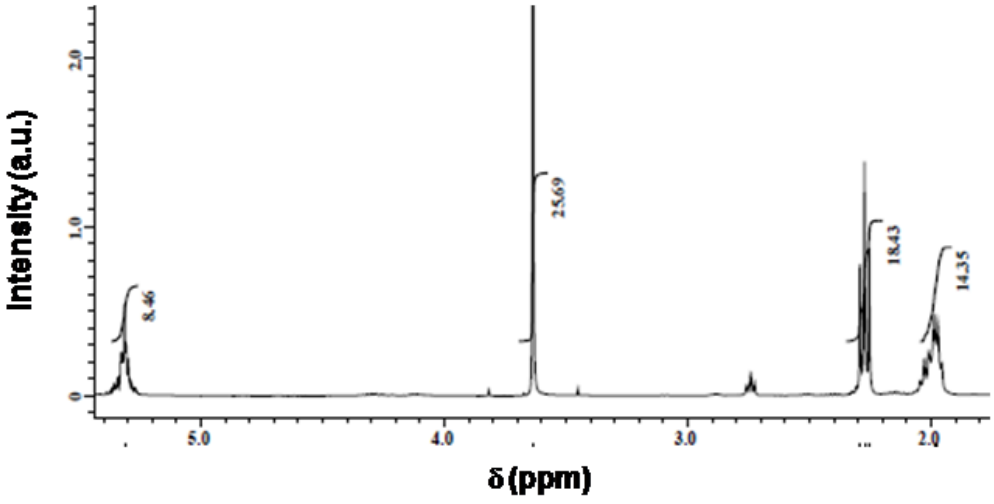


**Fig. 8.** $^{1}$H NMR spectra of fatty acid methyl ester (biodiesel) corresponding to the seed oil of *J. curcas* accession (TJS-01#04)

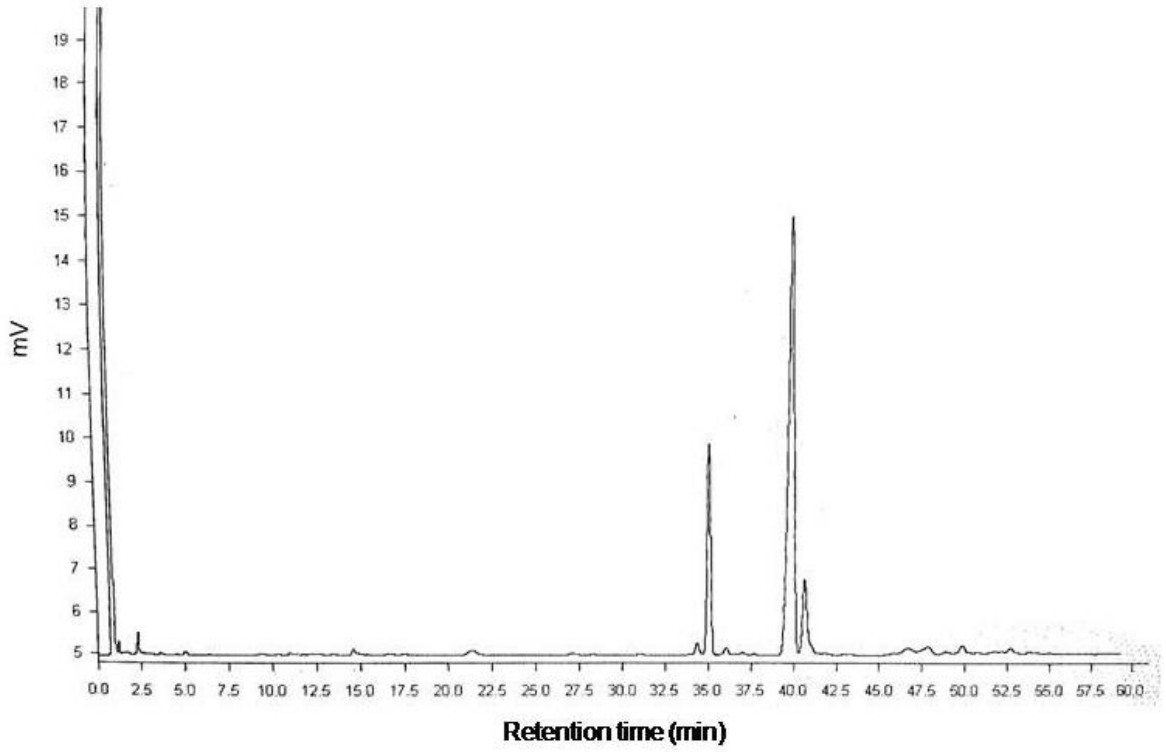


**Fig. 9.** GC chromatogram for the biodiesel sample (*J. curcas* accession TJS-01#04)

**Table 11:** Descriptive statistical analysis on the basis of fatty acid composition of the seed oil samples from the *J. curcas* accessions

| Fatty Acid | N | Mean | Minimum | Maximum | SD | Variance | Range |
|---|---|---|---|---|---|---|---|
| Palmitic Acid | 19 | 12.38 | 8.64 | 17.05 | 2.00 | 4.00 | 8.41 |
| Stearic Acid | 19 | 5.72 | 4.34 | 7.94 | 0.89 | 0.81 | 3.60 |
| Oleic Acid | 19 | 38.45 | 26.26 | 46.36 | 5.73 | 32.90 | 20.10 |
| Linoleic acid | 19 | 41.99 | 28.72 | 53.78 | 6.03 | 36.44 | 25.06 |

### 4.2.3 *Discussion*

Temporal and spatial expression of different developmentally-regulated fatty acid and lipid biosynthetic genes are known to influence the accumulation of storage lipids in the seeds of oil plants. In Jatropha (*J. curcas* L.), the storage lipids are mainly synthesized and accumulated in the seed endosperm. Gu et al. (2012) systemically investigated the expression of these genes at different developmental stages of *J. curcas* endosperm. It is likely that different *J. curcas*

accessions of the study could vary with regard to the expression patterns of these genes; consequently, influencing both quality and quantity of seed oil. As evident from several reports, various biometric and biochemical traits along with seasonal, agronomic and genetic factors influence seed yield, oil content, FAC, FFA concentration and other attributes in *J. curcas* (Kaushik and Bhardwaj, 2013; Sinha et al., 2015). 1H NMR spectra revealed that triacylglycerol (TAG) level was significantly low as compared to FFA at early developing stages of *J. curcas* seeds. There was a sharp decline of FFA level at the maturation stages as they are replaced by TAG, the predominant lipid in seeds (Annarao et al., 2008). In plants, diacylglycerol O-acyltransferase (DGAT) is committed to TAG biosynthesis and involved in plastidial fatty acid flux control in oil biosynthesis. There was a significant increase of oil content in both seeds and leaves in the transgenic *J. curcas* lines showing constitutive over expression of *AtDGAT1,* and extracted crude oil showed reduced FFA level in comparison to control wild-type plants (Maravi et al., 2016). A multitude of factors including stresses may likely to delay or interfere in the synthesis of fatty acids, TAG and other downstream pathways contributing to variation in FFA level in seeds of the chosen *J. curcas* accessions.

Apart from aforesaid metabolic flux control during seed development, other parameters like extraction process, improper handling, inappropriate storage condition, duration of storage, action of lipases may contribute to higher FFA level in seed oil. FFA content is recognized as one of the important physico-chemical properties with regard to quality of a vegetable oil. The value less than 1% is suitable for conversion to methyl esters; exceeding more than 2 wt% significantly compromised both the yield and recovery of biodiesel due to saponification during the process of base-catalyzed transesterification. High FFA content in *J. curcas* oil could be overcome by the two-step conversion process i.e., lowering FFA content by an acid-catalyzed esterification pretreatment prior to base-catalyzed transesterification. (Knothe, 2005; Tiwari et al., 2007; Berchmans and Hirata, 2008; Chai et al., 2014). Most of *J. curcas* seed oil samples of the study were found suitable in terms of low FFA content and efficient transesterification.

Five common major fatty acids of plant oils include palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and linolenate (18:3). FAC of a vegetable oil profoundly influences the quality of biodiesel; since the fuel properties such as fuel ignition quality, cold flow, heat of combustion, oxidative stability and viscosity depend mainly on the FAC of an oil, particularly high level of MUFA namely oleic acid (Knothe, 2005; Qu et al., 2012; Ullah et al., 2014).

Four major fatty acids namely palmitic, stearic, oleic and linoleic acids were found in all the *J. curcas* accessions, and their contents ranged from 8.64–17.05%, 4.34–7.94%, 26.26–46.36% and 28.72–53.78%, respectively. The lipid profile of *Jatropha Peiranoi* also showed the presence of linoleic acid (75%), oleic acid (15.6%), stearic acid (4%) and palmitic acid (5.9%) as primary fatty acids in the oil (Paterlini et al. 2019). The present study, the variances for the individual fatty acids are as follows: 4.0 for palmitic, 0.81 for stearic, 32.9 for oleic and 36.44 for linoleic acids. The total unsaturated fatty acid (UFA) content across the *J. curcas* accessions varied from 75.08–84.90% which were consistent with the Mexican accessions (59.14–86.76%), and the accessions collected in India (63.64–89.10%) as reported earlier (Ovando-Medina et al., 2011; Sinha et al., 2016). Based on FAC analysis, the *J. curcas* accessions could be distinctly grouped into two categories: a) oleic acid content was found to be more than linoleic acid in 9 oil samples, and the values ranged from 40.02–46.36%, b) high level of linoleic acid (more than 45%) was found in 6 oil samples. Various factors such as genotype, agronomic practices, edaphic and climatic conditions are considered to be the major contributing factors for variation of both seed oil content and FAC between the *J. curcas* accessions (Montes et al., 2015; Negussie et al., 2016). The impact of these factors could be understood through multi-location trials and performance evaluation of the individual accessions.

### *4.2.4 Conclusions*

Biodiesel has been identified as a promising renewable fuel source in many countries. In this study, seed oil content, oil quality in terms of FFA content and FAC were analyzed for selection of the superior *J. curcas* genotypes. The *J. curcas* accessions namely TJS–04#42, TJS–05#10, TJS–19#01, TJS–23#17, TJS–25#01, TJS–31#13, TJS–32#16 were quite promising in terms of oil content, FFA (≤1%, w/w) and fatty acid composition (Table 10). Most of these *J. curcas* accessions could be grouped into two categories: some of the seed oils were oleate-rich suitable for biodiesel feedstock; whereas others were linoleate-rich useful for other industrial applications. Apart from clonal propagation, field trials and large-scale plantations, these promising *J. curcas* accessions will be useful as prebreeding materials in various crop improvement programs.

## Objective-3: Micropropagation of elite clones of *Jatropha*

Currently, there is an emphasis on large scale cultivation of *J. Curcas* to meet the demand of biodiesel feedstocks; therefore, there is a requirement of developing improved varieties. Development of a simple, efficient, cost-effective and mass scale true-to-type propagation protocol through tissue is a prerequisite to produce and supply the quality planting material for large scale cultivation and field trials. Moreover, the tissue culture protocols are the basis for various genetic improvement programs through the application of biotechnological tools and have a potential to introduce desirable traits and for fast and large-scale multiplication of selected germplasm. In this study, a simple and improved micropropagation protocol for the elite *J. curcas* germplasm was developed through axillary shoot bud proliferation as described in the following sections.

## 4.3 Results and Discussion (Objective-3)

### *4.3.1 Explant Collection for In Vitro Culture Establishment*

Different types of explants from field-grown candidate plus trees (CPTs) responded well on MS medium supplemented with varying concentrations of cytokinins and auxins. Regular pruning of the CPTs on yearly basis (usually during December–January) helped us to get a higher number of juvenile explants. Explants harvested during the end of March to mid of July were found suitable for the establishment of the *in vitro* culture. Periodic minor pruning also helped in getting the nodal explants from the freshly grown sprouts. Incidence of contamination was observed more during the rainy season due to high humidity because of the tight packing of the dust particles/fungal spores/microbes in the harvested explants. This study mainly focused on one elite *J. curcas* accession i.e, TJS-01#04 for *in vitro* micropropogation study. The *J. curcas* nodal explants were collected from the field-grown plants as shown in Fig. 10 with necessary precautions, surface-sterilized and cultured on suitable media as described in the following sections.

### *4.3.2. Establishment of J. curcas Culture In Vitro*

Nodal explants from the CPTs cultured on MS medium lacking plant growth regulators (PGRs) showed very slow growth; but the growth response of the explants significantly changed with the addition of the individual PGRs like BAP, Kin, IBA, IAA, and TDZ either singly or in combination. Nodal explants and apical shoots had shown responses differently on the MS medium supplemented with varying concentrations of BAP, Kin, and TDZ. MS media supplemented with 0.25–2.0 mg/L of TDZ resulted in swelling at cut ends of nodal and shoot tip explants with the

only formation of a bunch of condensed adventitious shoot buds; no elongation was noticed by further sub-culturing on a medium supplemented with BAP alone or in combination with IBA (Fig. 11). The use of TDZ was found to be ineffective to produce shoot buds from nodal explants in *J. curcas*.


**Fig. 10.** Collection of nodal explants from field-grown *J. curcas* plant: (a) growing *J. curcas* plant at TIET campus; (b) growing juvenile shoot; (c) dressed shoots and (d) nodal explants.

**Fig.11.** Regeneration of condensed shoot buds on MS medium supplemented with TDZ (2.0 mg/L)

With regard to axillary shoot bud proliferation (Fig. 12a, b), the percentage response of nodal explants varied from 79.22 ± 5.77 to 100 ± 0.00 and 33.39 ± 6.67 to 97.01 ± 0.1 on BAP and Kn supplemented media, respectively. The number of shoot bud regeneration varied from 1.00 ± 0.12 to 1.98 ± 0.07 and 0.31 ± 0.06 to 1.52 ± 0.09; whereas, the length of the regenerated shoot buds varied from 0.56 ± 0.03 to 1.32 ± 0.05 cm and 0.16 ± 0.02 to 0.97 ± 0.09 cm containing BAP and Kn, respectively. MS medium supplemented with 2.0 mg/L BAP was found to be most effective in terms of percentage explant response (95.56 ± 3.72), number of shoot buds (1.84 ± 0.06) and shoot length (1.32 ± 0.05 cm) per explant within four weeks. For BAP, more or less similar responses were reported earlier (Rathore et al. 2015). Higher concentration of BAP (5.0 mg/L) resulted in higher bud breaks; but with the progress of time, shoot buds faced hyper-hydration and profuge callusing alongside the base and cut ends of the explants. In the case of MS medium supplemented with Kn, we noticed delayed explant response, appearance of weak and pale yellow proliferated shoots with reduced vigor. The cytokinins BAP and Kn used in combination led to an increase in the number of shoot buds from lower to higher concentrations (1.0–5.0 mg/L) with gradual reduction of mean shoot length (Table 12).

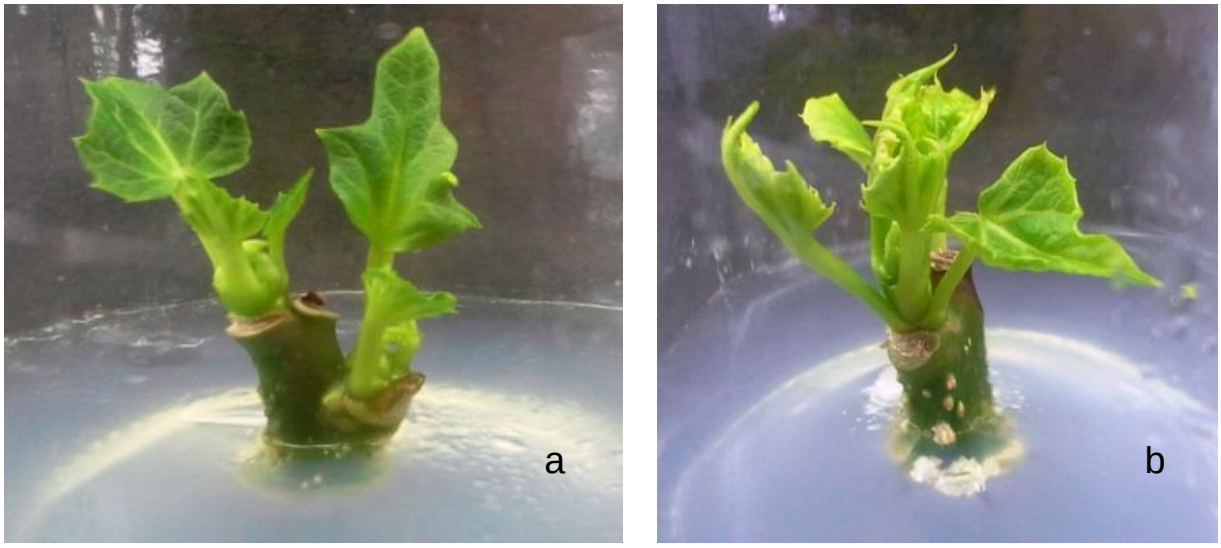


**Fig. 12.** Bud break from two (a, b) nodal explants from *J. curcas* accession, TJS-04 #42.

**Table 12:** Effect of plant growth regulators (BAP and Kin) on the proliferation of axillary meristem from the nodal explants of *J. curcas*

| PGRs | Concentration (mg/L) | Percent response ± S.D | Average number of shoot buds ± S.D | Average length (cm) of shoot buds ± S.D |
|---|---|---|---|---|
| Control | 0.0 | $26.33 \pm 1.04^{f}$ | $0.28 \pm 0.01^{f}$ | $0.08 \pm 0.01^{f}$ |
| BAP | 0.5 | $79.22 \pm 5.77^{c}$ | $1.00 \pm 0.12^{d}$ | $0.56 \pm 0.03^{c}$ |
| | 1.0 | $85.44 \pm 6.63^{bc}$ | $1.42 \pm 0.13^{bc}$ | $0.85 \pm 0.01^{b}$ |
| | 1.5 | $89.23 \pm 4.13^{b}$ | $1.61 \pm 0.05^{b}$ | $1.10 \pm 0.04^{a}$ |
| | 2.0 | $95.56 \pm 3.72^{ab}$ | $1.84 \pm 0.06^{a}$ | $1.32 \pm 0.05^{ab}$ |
| | 3.0 | $94.37 \pm 3.33^{a}$ | $1.70 \pm 0.12^{b}$ | $1.21 \pm 0.07^{a}$ |
| | 5.0 | $100 \pm 0.00^{a}$ | $1.98 \pm 0.07^{a}$ | $0.71 \pm 0.03^{bc}$ |
| Kinetin | 0.5 | $33.39 \pm 6.67^{de}$ | $0.31 \pm 0.05^{e}$ | $0.16 \pm 0.02^{e}$ |
| | 1.0 | $47.23 \pm 8.82^{d}$ | $0.40 \pm 0.06^{e}$ | $0.33 \pm 0.03^{d}$ |
| | 1.5 | $66.85 \pm 5.77^{c}$ | $0.82 \pm 0.08^{d}$ | $0.55 \pm 0.01^{c}$ |
| | 2.0 | $85.55 \pm 3.21^{b}$ | $1.19 \pm 0.06^{d}$ | $0.80 \pm 0.07^{bc}$ |
| | 3.0 | $92.13 \pm 4.77^{a}$ | $1.31 \pm 0.05^{cd}$ | $0.97 \pm 0.06^{b}$ |
| | 5.0 | $97.01 \pm 0.1^{a}$ | $1.52 \pm 0.12^{bc}$ | $0.93 \pm 0.02^{b}$ |

Values are mean ± S.D, and values followed by different letters in the columns are significant at $P \leq 0.05$

### *4.3.3 Culture amplification and shoot bud elongation*

Multiplication of *J. curcas* shoot buds was carried out by repeated transfer and sub-culture of the nodal segments produced *in vitro*. For this purpose, both cytokinin and auxin were used at relatively lower concentartions as compared to the establishment of the cultures as stated earlier. MS medium supplemented with BAP 0.5 mg/L and IBA 0.25 mg/L was found to be most effective.

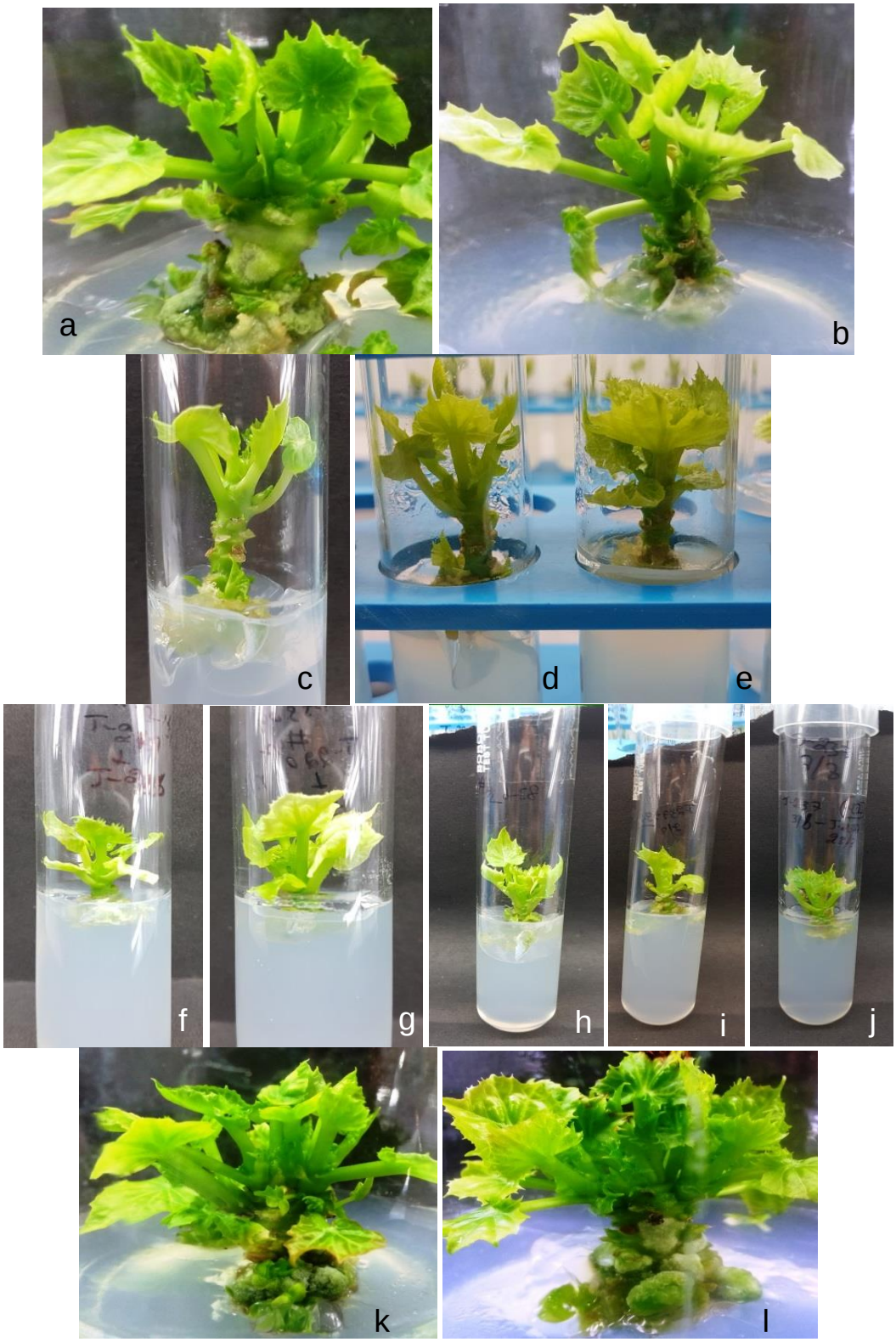


**Fig. 13.** Micropropagation of the elite *J. curcas* accession: (a, b) Multiplication of shoot buds by repeated transfer to the fresh medium; (c–e) Elongated shoots; (f–j) Amplification of cultures by culturing *in vitro* derived nodal segments; (k–l) Axillary shoot bud proliferation from *in vitro* derived nodes.

Periodic transfer to this medium resulted in considerable improvement of *in vitro* nodal segments in terms of both shoot number and length. After 4 weeks of growth for each transfer, the values of shoot number and length were found to be 2.43 ± 0.15 shoots/2.54 ± 0.19 cm, 3.81 ± 0.19 shoots/2.92 ±0.09 cm, and 4.92 ± 0.25 shoots/3.13 ± 0.07 cm after first, second and third transfer, respectively (Table 13). Overall, the media effectively worked with regard to production of healthy, juvenile and green shoots ((Fig. 13a, b).

**Table 13:** Effect of different concentrations and combinations of BAP and IBA on shoot bud multiplication *in vitro* cultures by repeated transfer of newly regenerated shoot buds of *J. curcas*

| PGR (mg/L) | | Transfer of shoot segments | | | | | |
|---|---|---|---|---|---|---|---|
| | | First | | Second | | Third | |
| BAP | IBA | Average number of shoot buds | Average length (cm) of shoot buds | Average number of shoot buds | Average length (cm) of shoot buds | Average number of shoot buds | Average length (cm) of shoot buds |
| 0.5 | 0.1 | $1.81 \pm 0.12^{de}$ | $1.41 \pm 0.17^{cde}$ | $2.32 \pm 0.13^{d}$ | $2.13 \pm 0.12^{ab}$ | $2.17 \pm 0.15^{e}$ | $2.31 \pm 0.15^{bc}$ |
| 0.5 | 0.25 | $2.43 \pm 0.15^{cd}$ | $2.54 \pm 0.19^{b}$ | $3.81 \pm 0.19^{c}$ | $2.92 \pm 0.09^{a}$ | $4.92 \pm 0.25^{b}$ | $3.13 \pm 0.07^{a}$ |
| 0.5 | 0.5 | $2.53 \pm 0.25^{cd}$ | $2.93 \pm 0.24^{a}$ | $3.52 \pm 0.21^{c}$ | $2.69 \pm 0.17^{a}$ | $4.23 \pm 0.19^{c}$ | $2.94 \pm 0.09^{a}$ |
| 0.5 | 1.0 | $2.67 \pm 0.30^{cd}$ | $2.99 \pm 0.30^{a}$ | $3.89 \pm 0.10^{c}$ | $2.62 \pm 0.13^{b}$ | $4.31 \pm 0.13^{c}$ | $2.15 \pm 0.05^{cd}$ |
| 1.0 | 0.1 | $2.32 \pm 0.15^{d}$ | $1.32 \pm 0.26^{cde}$ | $2.53 \pm 0.21^{d}$ | $1.24 \pm 0.14^{def}$ | $2.83 \pm 0.13^{d}$ | $1.71 \pm 0.21^{cde}$ |
| 1.0 | 0.25 | $2.42 \pm 0.07^{cd}$ | $1.65 \pm 0.19^{d}$ | $2.54 \pm 0.21^{d}$ | $1.72 \pm 0.21^{cde}$ | $2.72 \pm 0.17^{d}$ | $1.83 \pm 0.06^{cd}$ |
| 1.0 | 0.5 | $2.57 \pm 0.18^{cd}$ | $1.73 \pm 0.18^{cd}$ | $3.85 \pm 0.23^{c}$ | $1.73 \pm 0.11^{cde}$ | $4.71 \pm 0.15^{c}$ | $1.89 \pm 0.15^{cd}$ |
| 1.0 | 1.0 | $2.92 \pm 0.12^{bc}$ | $1.75 \pm 0.12^{cd}$ | $4.36 \pm 0.09^{b}$ | $1.54 \pm 0.07^{de}$ | $5.19 \pm 0.11^{ab}$ | $1.72 \pm 0.09^{cde}$ |
| 2.0 | 0.1 | $2.83 \pm 0.12^{bc}$ | $1.62 \pm 0.23^{d}$ | $4.65 \pm 0.31^{ab}$ | $1.73 \pm 0.13^{de}$ | $5.27 \pm 0.09^{ab}$ | $1.25 \pm 0.02^{de}$ |
| 2.0 | 0.25 | $3.72 \pm 0.15^{b}$ | $1.64 \pm 0.17^{d}$ | $4.92 \pm 0.29^{a}$ | $1.67 \pm 0.09^{de}$ | $5.47 \pm 0.11^{ab}$ | $1.66 \pm 0.15^{cde}$ |
| 2.0 | 0.5 | $4.13 \pm 0.18^{a}$ | $1.87 \pm 0.15^{cd}$ | $4.97 \pm 0.26^{a}$ | $1.58 \pm 0.11^{de}$ | $5.63 \pm 0.19^{a}$ | $1.52 \pm 0.13^{de}$ |
| 2.0 | 1.0 | $4.26 \pm 0.17^{a}$ | $1.91 \pm 0.11^{cd}$ | $5.23 \pm 0.34^{a}$ | $1.42 \pm 0.16^{e}$ | $5.75 \pm 0.23^{a}$ | $1.43 \pm 0.07^{de}$ |

Values are mean ± S.D; and values followed by different letters in the columns are significant at $P \leq 0.05$

Further, the *in vitro* grown healthy *J. curcas* shoots were carefully excised and cultured individually on the elongation medium (Fig. 13c–e). The elongated shoots were further sub-cultured by cutting them into the apical shoot portion and nodal segments (Fig. 13f–j). The nodal segments from the *in vitro* grown plantlets were further multiplied by transfer to the same the repeated subculture of *in vitro* derived nodal segments (Fig. 13k, l). The timing of the transfer of *in vitro* derived nodal segments to fresh medium after every 4 weeks remained crucial as delay in shifting led to hyperhydration and yellowing of the shoot buds.

MS medium supplemented with 0.5 mg/L BAP and 0.25 Mg/L IBA leads to the formation of 8.66 ± 0.21 shoots of 2.21 ± 0.04 cm length from *in vitro* derived nodal segments within four (4) wks indicating 1:8.6-fold of shoot multiplication. Further, these shoots achieved elongation of 2.75 ±

0.05 cm in first, 3.31 ± 0.03 cm in second, and 4.97 ± 0.03 cm height in the third transfer (Table 14). The first root-able *J. curcas* shoot from the CPT-derived explant was achieved after 16 wks with 1:4.92-fold of shoot multiplication.

Although, MS medium supplemented with relatively higher concentrations of BAP (1.0–2.0 mg/L in combination with IBA (0.25–1.0 mg/L) (Table 14) resulted in an increase in the number of shoot buds, but, the newly formed shoot buds showed stunted growth with hyperhydration with consecutive transfers (data not shown).

**Table 14:** Effect of different concentrations and combinations of BAP and IBA on multiple shoot bud induction from *in vitro* derived nodal segments and subsequent elongation of shoots of *J. curcas*

| PGR conc. (mg/L) | | Shoot bud induction from in vitro derived nodal segments | | Elongation of shoot buds regenerated from in vitro derived nodal segments | | |
|---|---|---|---|---|---|---|
| BAP | IBA | Average number of shoot buds | Average length (cm) of shoot buds | Transfer-1 | Trasfer-2 | Transfer-3 |
| 0.25 | 0.0 | $2.62 \pm 0.11^{f}$ | $1.73 \pm 0.03^{bc}$ | $2.35 \pm 0.04^{bc}$ | $2.63 \pm 0.06^{c}$ | $2.69 \pm 0.06^{e}$ |
| | 0.25 | $3.24 \pm 0.17^{e}$ | $1.89 \pm 0.05^{b}$ | $2.51 \pm 0.05^{b}$ | $2.72 \pm 0.06^{c}$ | $2.82 \pm 0.07^{cde}$ |
| | 0.5 | $3.91 \pm 0.12^{de}$ | $2.12 \pm 0.09^{ab}$ | $2.62 \pm 0.08^{ab}$ | $2.79 \pm 0.07^{bc}$ | $3.05 \pm 0.07^{cd}$ |
| | 1.0 | $4.75 \pm 0.19^{cd}$ | $1.89 \pm 0.01^{b}$ | $2.69 \pm 0.04^{a}$ | $2.73 \pm 0.09^{c}$ | $3.01 \pm 0.07^{cd}$ |
| 0.5 | 0.0 | $6.82 \pm 0.09^{c}$ | $2.01 \pm 0.07^{ab}$ | $2.61 \pm 0.05^{ab}$ | $2.82 \pm 0.05^{ab}$ | $2.91 \pm 0.04^{cde}$ |
| | 0.25 | $8.66 \pm 0.21^{a}$ | $2.21 \pm 0.04^{a}$ | $2.75 \pm 0.05^{a}$ | $3.31 \pm 0.03^{a}$ | $4.97 \pm 0.03^{a}$ |
| | 0.5 | $8.13 \pm 0.11^{a}$ | $2.01 \pm 0.09^{ab}$ | $2.72 \pm 0.01^{a}$ | $3.01 \pm 0.07^{a}$ | $3.92 \pm 0.06^{b}$ |
| | 1.0 | $7.92 \pm 0.18^{b}$ | $1.93 \pm 0.02^{b}$ | $2.72 \pm 0.01^{a}$ | $2.89 \pm 0.09^{ab}$ | $3.31 \pm 0.06^{cd}$ |

Values are mean ± S.D; and values followed by different letters in the columns are significant at $P \leq 0.05$

### *4.3.4 Root Regeneration*

For rooting of the *in vitro* grown excised shoots, ½ strength of MS media supplemented with the different concentrations of root inducing PGRs were used. Only ½ strength MS medium lacking PGR did not show any sign of rooting of shoots. For rooting purpose, ½ strength MS supplemented with 3.0 mg /L IBA effectively worked in this study as shown in Fig. 14a . In this media formulation, 75.12 ± 4.33 shoots responded and produced 3.38 ± 0.09 roots of 4.29 ± 0.09 cm length within four (4) weeks. Root regeneration was found to vary with the application of different root hormones; the values of root regenartion were calculated as follows: 33.33 ± 5.88 to 76.12 ± 5.89 (IBA at different conc.); 21.97 ± 3.33 to 50.66 ± 4.33 (IAA at different conc.) and 33.33 ± 3.33 to 50.36 ± 4.33 (NOA at different conc.). The respective number of roots were as follows: 0.54 ± 0.07 to 3.16 ± 0.08 (IBA), 0.13 ± 0.09 to 2.11 ± 0.11 (IAA) and 0.34 ± 0.08 to 2.27 ± 0.13

(NOA) supplemented medium and the root length ranged from 1.73 ± 0.02 to 3.71 ± 0.17 for IBA, 0.77 ± 0.13 to 1.98 ± 0.11 for IAA and 0.62 ± 0.02 to 1.83 ± 0.07 for NOA (Table 15).


**Fig. 14.** Rooting and acclimatization of *in vitro* raised *J. curcas* plants: (a) *In vitro* rooting; (b) Acclimatized plantlets.

**Table 15:** Effect of different concentrations of IBA, IAA, and NOA on in vitro root induction from the cloned shoot of *J. curcas* on 1/2 strength of MS medium

| PGRs | Concentration (mg/L) | Percent response | Average number of roots | Average length (cm) of roots |
|---|---|---|---|---|
| Control | 0.0 | 0.0 | 0.0 | 0.0 |
| IBA | 1.0 | $33.33 \pm 5.88^{e}$ | $0.54 \pm 0.07^{ef}$ | $1.73 \pm 0.02^{de}$ |
| | 2.0 | $45.47 \pm 4.33^{c}$ | $1.23 \pm 0.16^{d}$ | $2.65 \pm 0.13^{cd}$ |
| | 3.0 | $75.12 \pm 4.33^{a}$ | $3.38 \pm 0.09^{a}$ | $4.29 \pm 0.09^{a}$ |
| | 5.0 | $76.12 \pm 5.89^{a}$ | $3.16 \pm 0.08^{a}$ | $3.71 \pm 0.17^{b}$ |
| IAA | 1.0 | $21.97 \pm 3.33^{f}$ | $0.13 \pm 0.09^{f}$ | $0.77 \pm 0.13^{f}$ |
| | 2.0 | $33.21 \pm 3.21^{e}$ | $0.54 \pm 0.13^{ef}$ | $1.21 \pm 0.09^{e}$ |
| | 3.0 | $45.07 \pm 4.77^{c}$ | $1.35 \pm 0.15^{d}$ | $1.57 \pm 0.17^{de}$ |
| | 5.0 | $50.66 \pm 4.33^{c}$ | $2.11 \pm 0.11^{bc}$ | $1.98 \pm 0.11^{d}$ |
| NOA | 1.0 | $33.33 \pm 3.33^{e}$ | $0.34 \pm 0.08^{ef}$ | $0.62 \pm 0.02^{f}$ |
| | 2.0 | $41.49 \pm 4.21^{c}$ | $0.92 \pm 0.10^{de}$ | $1.12 \pm 0.11^{e}$ |
| | 3.0 | $49.77 \pm 6.66^{c}$ | $1.67 \pm 0.22^{cd}$ | $1.68 \pm 0.09^{de}$ |
| | 5.0 | $50.36 \pm 4.33^{d}$ | $2.27 \pm 0.13^{bc}$ | $1.83 \pm 0.07^{d}$ |

Values are mean ± S.D, and values followed by different letters in the columns are significant at $P \leq 0.05$

### *4.3.5 Acclimatization and transplantation in soil*

The *in vitro* raised rooted *J. curcas* plantlets as shown in Fig. 14a were acclimatized by slow and gradual exposure from high relative humidity (RH) and low-temperature conditions to low RH and

high-temperature conditions. ¼ strength MS medium was added as a nutrient solution to the plantlets. In order to control fungal contamination, 1.0 g/L Bavistin was sprayed on the rooted plantlets during the process of acclimatization.. The controlled exposure of these plantlets from different temperatures and humidity regimes during shifting from *in vitro* to field conditions only ensured their survival and adaptation to growth under environmental conditions. The hardened plants were first planted in larger polybags, followed by its plantation in the pots and field (Fig. 14b), and a limited study was carried out to notice their growth charcetrictics.

### *4.3.6 Discussion*

*J. curcas* genotypes propagated through seeds have shown significant variations in seed yield and seed oil content because a) monoecious, b) heterozygosity, c) transmission of seed-borne diseases to the seedling, d) edaphic factors, e) uncertain fruit yield, and f) seed viability. The genotypes propagated through vegetative cuttings also have many limitations due to a) availability of sufficient quantity of quality planting materials, b) seasonal barriers, c) pseudo-taproot system, d) lower longevity, e) low tolerance to drought and disease, f) less seed yield, and g) carryover of disease-causing pathogens from one generation to next. Therefore, conventional propagation of *J. curcas* crop through seed raised plants is not preferred; at the same time, vegetative propagation through cutting raised plants has an inherent lacunae to meet the demand of large amounts quality planting materials round the year (Heller 1996; Openshaw 2000; Sujatha et al. 2005; Divakara et al. 2010). Establisment of superior *J. curcas* germplasm *in vitro* and development of easy-to-do micropropagation protocols are important aspects for mass multiplication and genetic improvement through applications of modern biotechnological tools.

The candidate plus trees (CPTs) under field conditions face several kinds of stresses which affect the physiological status and overall seed yield/oil content/oil quality of the plants as they are influenced by both exogenous and endogenous signals. Such type of stresses stresses could be overcome to a considerable extent by regular monitoring and maintenance of the planted trees by several agricultural practices in the form of timely irrigation, weeding, application of farmyard manure (FYM), and proper pruning. These steps ensure the availability of the juvenile explants for the *in vitro* culturing from the healthy CPTs found at different locations (Srivastava et al. 2011; Rathore et al. 2015).

An extensive survey, analysis of the seed traits, seed oil content and fatty acid analysis (FAC) helped us identify a number of superior *J. curcas* genotypes in the state of Punjab, India as reported

earlier (Kumar and Das 2018a; Kumar and Das 2018b). Only, a few of these promising *J. curcas* genotypes were used for micropropagation in order to generate true to-to-type propagules under laboratory conditions. The main purpose was to produce more number of clones of the individual accessions.

Explants like nodal segments and shoot tips have shown better response on the media containing cytokinins and auxins. For regenartion under *in vitro* conditions, healthy juvenile explants showed better response as compared to the hard and mature shoot segments. Use of TDZ at different concentrations resulted in massive swelling with callus formation at the cut ends of the explants and subsequently produced bunch of condensed adventitious shoot buds which failed to elongate during further subculturing on media supplemented with cytokinins in combination auxins. There are some reports where we find the use of TDZ as a cytokinin for *J. curcas* culture *in vitro* (Sujatha et al. 2005 Datta et al. 2007; Singh et al. 2010; Khurana-kaul et al. 2010). However, in the present study, TDZ did not work effectively, and therefore skipped from the media formulations.

As stated earlier in the Result section, MS medium supplemented with BAP 0.5 mg/L and IBA 0.25 mg/L was found to be most effective for shoot growth and multiplication form *J. curcas* nodal explants. The results were found to be consistent with the earlier reports (Sujatha et al. 2005; Datta et al. 2007; Singh et al. 2010). Lower concentration of BAP (0.25 mg/L) led to regeneration of slim, hardy shoots with early maturation characteristics, and nodal segments produced from these shoots produced less number of shoot buds on repeated transfers. Higher concentration IBA (1.0 mg/L) induced vitrification of shoot buds if applied with varying BAP levels (0.25–0.5 mg/L). Paterlini et al. (2019) also reported the effevtiveness of the BAP in terms of shoot bud induction and subsequent proliferation from nodal explants of *Jatropha peiranoi* at low temperature. Adventitious shoot bud induction from stem explants of *J. curcas* was observed by using combinations of BAP and Kn in the culture medium (Singh et al. 2010). Earlier, Singh (2018) reported the shoot bud induction from nodal segments in the liquid MS medium supplemented with Kn 2.0 mg/L. However, in this study, Kn was found to be less effective in comparison to BAP with regard to shoot bud induction in *J. curcas* because of the following contraints: slow explant response, poor regeneration of shoot buds in terms of both number and growth pattern. All these results support a common view that BAP is more effective in comaprision to other cytokinins with regard to *J. curcas* micropropagation.

Multiplication of *J. curcas* shoot buds involved, repeated transfer and subculture of the *in vitro* derived nodal segments. Repeated transfer of explants was carried out *in vitro* in a time-dependent manner to promote rejuvenation and activation of meristems for the production of more number of healthy shoot buds. Repeated culturing of the *J. curcas* explants to fresh media helped in retention of the morphogenetic potential as reported earlier (Sujatha et al. 2005; Singh et al. 2009, Rathore et al. 2015). Use of different types of vessels also had a significant effect on culture maintenance and multiplication (Khurana-Kaul et al. 2010).

After establishment of *J. curcas* culture *in vitro*, nodal segments from the *in vitro* derived shoots were used for the proliferation of new shoots in the next regeneration cycle. What it meant, a cyclic way of shoot bud multiplication protocol was established. The synergistic effect of BAP and IBA was also observed in shoot bud regeneration and further growth of the regenerated shoots *in vitro*. Rathore et al. (2015) reported that BAP in combination with IAA had showed improvement significantly in shoot multiplication from the nodal segments in terms of both shoot number and length. Shoot proliferation and elongation were observed in liquid MS media supplemented with BAP (1.5 mg/L), IAA (0.5 mg/L) and Kn (0.2 mg/L) (Singh 2018).

Clonally pure shoots raised under *in vitro* culture conditions were transferred to the root induction medium. IBA (3.0 mg/L) was found to be effective for regeneration *in vitro.* Similar results were also reported earlier in *J. curcas* (Datta et al. 2007; Singh et al. 2010; Khurana-Kaul et al. 2010; Joshi et al. 2011, Rathore et al. 2015). Root regeneration was also reported only in MS media without auxin supplements *(*Purkayastha et al. 2010*). In vitro* raised *J. curcas* plantlets were effectively acclimatized under controlled conditions prior to shifting to the field condition. Use of antibiotic like Augmentin has been reported for better survival rate and growth of plantlets during acclimatization (Toppo et al. 2012). The micropropagation protocol as adopted in this study could be employed for mass multiplication of other elite *J. curcas* accessions characterized in this study and also in genetic improvement programmes.

### *4.3.7* Conclusions

In conclusion, a simple, efficient, and true-to-type *in vitro* clonal propagation protocol was developed in select *J. curcas* accession(s) using axillary shoot buds. This method was reproducible which would help in producing genetically uniform and disease-free healthy plantlets throughout the year despite seasonal variations; quality planting material of *J. curcas* could be raised for field trials, genetic improvement, and large-scale plantations.

## 5. Summary

- An extensive survey was carried out to select a total of 31 morphologically superior candidate plus trees (CPTs) of *J. curcas* from different locations in the state of Punjab, a North Western part of India having latitude, longitude and altitude ranging 30°20′–31°32′N, 74°18′–76°28′E, and 211−260 m, with average annual rainfall about 700 ± 50 mm, respectively.
- From each CPT, mature black fruits were harvested during the months of December and January, and the seeds were collected through the dehulling and shelling processes, dried under the sun for 1-2 days till they attained constant weight. The seeds of the individual *J. curcas* CPTs showed considerable variation with regard to shape, size, and color of the coats.
- The range of 100-seed weight varied markedly from 35.10 g (TJS-02#01) to 77.34 g (TJS-31#13). For the majority of the accessions, the values were found to be in the range of 60-70 g; however, there were 6 accessions that showed the values above this range. The *J. curcas* accessions namely TJS–04#42, TJS–05#10, TJS–19#01, TJS–23#17, TJS–25#01, TJS–42#01, TJS–46#04 were quite promising in terms of both 100-seed weight and importantly, more than 35% oil content.
- The *J. curcas* seed oil % on kernel basis was determined using Soxhlet apparatus. Seed oil content was found to vary from 13.74% (TJS-02#01) to 54.37% (TJS-04#42). 24 accessions showed 30-40% oil content; 3 accessions showed more than 40% oil content, and for the remaining, it was less than 30%.
- Positive and significant correlation was observed between 100-seed weight and oil content (r = 0.517). The trait like seed length was found to be positively correlated with 100-seed weight, oil content, and seed width.
- The Free fatty acid (FFA) content of each seed oil sample was determined by the titrimetric method. Briefly, 10 g of oil sample was dissolved in a mixture of ethanol and diethyl ether (1:1 volume ratio) and titrated with 0.1 M KOH solution using phenolphthalein as an indicator. The FFA content in the seed oil ranged from 0.21–1.82% for most of the *J. curcas* accessions.
- The extracted *J. curcas* seed oils were efficiently transesterified to fatty acid methyl esters by treatment with methanol in presence of KOH. Proton nuclear magnetic resonance ($^1$H NMR) method was employed to assess the quality of the biodiesels i.e., fatty acid methyl esters

(FAMEs) on the basis of their FFA contents. The *J. curcas* accessions namely TJS–04#42, TJS–05#10, TJS–19#01, TJS–23#17, TJS–25#01, TJS–31#13, TJS–32#16 were quite promising in terms of oil content, FFA (≤1%, w/w) and fatty acid composition.

- The fatty acid compposition (FAC) of the FAME samples were carried out by gas chromatography. The contents of the four major fatty acids were found to vary significantly in the seed oils viz palmitic acid (8.64–17.05%), stearic acid (4.34–7.94%), oleic acid (26.26–46.36%) and linoleic acid (28.72–53.78%). Some of the seed oils were oleate-rich suitable for biodiesel feedstock; whereas others were linoleate-rich useful for other industrial applications.
- The explants for *in vitro* tissue culture were taken from the *J. curcas* CPTs maintained in the nursery at Thapr Technology Campus, Patiala. Murashige and Skoog's (MS) basal medium was used. A simple and reproducible micropropagation protocol was adopted for *in vitro* clonal propagation of the select *J. curcas* accessions through axillary shood bud proliferation from nodal segments. MS medium supplemented with BAP (2.0 mg/L) was found to be effective in terms of percentage explant response (95.56 ± 3.72 %), number of shoot buds (1.84 ± 0.06) and shoot length (1.32 ± 0.05 cm) per explant.
- Multiplication of *J. curcas* shoot buds i.e., 8.66 ± 0.21 shoots of 2.21 ± 0.04 cm length per nodal segment was carried out by repeated transfer and sub-culture of the nodal segments produced *in vitro* on MS medium supplemented with BAP 0.5 mg/L and IBA 0.25 mg/L.
- For the purpose of rooting *in vitro*, ½ strength MS supplemented with 3.0 mg /L IBA effectively worked in this study. It was noticed that 75.12 ± 4.33 % shoots responded and produced 3.38 ± 0.09 roots of 4.29 ± 0.09 cm length. The *J. curcas* plantlets raised *in vitro* were acclimatized by slow and gradual exposure from high RH and low-temperature to low RH and high-temperature conditions. The hardened plants were first transferred to large polybags followed by their planting in the pots under field condition.

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# 7. Publications

## ***Published Research Articles in Peer-Reviewed Journals***

- Kumar R, Das N (2018) Survey and selection of *Jatropha curcas* L. germplasm: Assessment of genetic variability and divergence studies on the seed traits and oil content. Ind Crops Prod 118:125–130
- Kumar R, Das N (2018) Seed oil of *Jatropha curcas* L. germplasm: Analysis of oil quality and fatty acid composition. Ind Crops Prod 124:663–668

## ❖ ***Abstracts Published in the Conference Proceedings***

- Aggarwal A, Garg R, Kumar R, Das N (2009) Molecular cloning studies on seed-expressed gene promotors from oilseed brassica crops. Biotech-2009 Present and future perspectives, Punjabi University, Patiala
- Das N, Aggarwal A, Kumar R, Aminedi R (2010) Molecular approaches for isolation and characterization of seed-specific gene promotors from different oilseed cultivars of Brassica. 4$^{th}$ Annual convention of association of biotechnology and pharmacy, Thapar University, Patiala
- Kaur G, Kumar R, Das N (2018) Soyabean waste okara: A promising feedstock for biodegradable plastics. 5$^{th}$ World congress on green chemistry and green engineering
- Kumar R, Das N (2019) *Jatropha curcas* L.: A promising tree-borne oilseed crop for biodiesel production.41$^{st}$ Annual Meeting of the Plant Tissue Culture Association of India, Thapar Institute of Engineering & Technology, Patiala

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# Survey and selection of *Jatropha curcas* L. germplasm: Assessment of genetic variability and divergence studies on the seed traits and oil content

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ABSTRACT

*Jatropha curcas* L., member of the *Euphorbiaceae* family, is widely recognized as a promising bioenergy crop. Its seed oil is used particularly for eco-friendly biodiesel production. A thorough survey led to select a total of 31 morphologically superior candidate plus trees (CPTs) of *J. curcas* from different locations of Punjab, a North-Western state of India. The seed samples showed considerable variation with regard to shape, size and color of the seed coats. 100-seed weight ranged from 35.10–77.34 g. Seed oil content was found to vary from 13.74% to 54.37%. Most of the accessions showed 30–40% oil content, and a few accessions showed more than 40% oil content. Both genotypic and phenotypic variances were highest and comparable for 100-seed weight followed by seed oil content. Genotypic coefficient of variation and phenotypic coefficient of variation also showed similar pattern. The highest heritability of 99% was recorded for 100-seed weight followed by oil content (97%) and seed length (81%). Positive and significant correlation was observed between 100-seed weight and oil content (r = 0.517). The *J. curcas* accessions were distinctly grouped into 6 clusters on the basis of non-hierarchical *K*-Means cluster analysis. Some of these accessions will be useful in terms of germplasm exchange, mass propagation, multi-location trials, feedstocks for biodiesel and as prebreeding materials for genetic improvement.

## 1. Introduction

Globally, rapid urbanization, industrialization, massive transportation and many other man-made activities not only lead to depletion of conventional and non-renewable fossil fuels but also significantly compromised the environmental health through emission of greenhouse gases (GHG). Eco-friendly biofuels namely bio-diesel and bio-ethanol have become focus areas of active research during the last few decades (Agarwal, 2007; Mukherjee et al., 2011). Some of the important non-edible tree borne oilseeds (TBOs) in Indian sub-continent include Neem (*Azadirachta indica*), Karanj (*Pongamia pinnata*), Mahua (*Madhuca indica*), *Jatropha* (*Jatropha curcas*), which produce seed oils suitable for biodiesel production. They are perennial in nature and continue to yield for a few decades. These non-edible TBOs can grow under different agro-climatic and edaphic conditions covering non-agricultural and waste land areas (Dhyani et al., 2015). *Jatropha curcas* L. or physic nut ($2n = 2x = 22$), member of the *Euphorbiaceae* family and native to Central America and Mexico, is a multipurpose deciduous small tree or shrub. It is now distributed in many tropical and sub-tropical regions of Africa and Asia. *J. curcas* draws the attention of many researchers for their rapid growth, easy propagation, drought tolerance, pest resistance, and most importantly high seed yield and oil content which are prerequisites for biodiesel production (Heller, 1996; Openshaw, 2000; Fairless, 2007).

During the last more than two decades, the major focus areas of research on the bioenergy crop *J. curcas* include: a) systematic survey, selection of candidate plus trees (CPTs), characterization and evaluation of *Jatropha* germplasm for the traits such as growth, morphology, seed characteristics, seed yield, oil content (Kaushik et al., 2007; Rao et al., 2008; Mishra, 2009;Tripathi et al., 2013; Duong et al., 2013; Francis et al., 2017), b) development and use of different molecular markers for assessment of genetic variability (Basha and Sujatha, 2009; Pioto et al., 2015), c) mass propagation, multi-location trials, performance evaluation, assessing genetic variation in different environments, development of agro-technologies (Srivastava et al., 2011; Singh et al., 2013), d) genetic improvement through mass and recurrent selection, mutation and heterosis breeding, molecular breeding, and inter-specific hybridization (Divakara et al., 2010; Kumar et al., 2015), e) proteome and transcriptome analysis, isolation and characterization of different genes, functional genomics (Yang et al., 2009; Costa et al., 2010; Maghuly and Laimer, 2013), and f) micropropagation, development of transformation protocols and transgenics (Mukherjee et al., 2011; Rathore et al., 2015). As evident from these studies, *Jatropha* can adapt to a wide range of environmental conditions. The potential of

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seed yield and oil content are determined by environmental, genetic and physiological factors. Large-scale production of biodiesel from various vegetable oils including *J. curcas* through transesterification has also become an important area of research in many laboratories. In this process, triacylglycerol (TAG) molecules react with an alcohol namely methanol or ethanol in the presence of a catalyst to form fatty acid methyl/ethyl esters and glycerol. Various parameters such as oil quality, nature of catalysts, process conditions, reactor/separator design influence the transesterfication process (Klofutar et al., 2010; Wang et al., 2011; Likozar and Levec, 2014; Likozar et al., 2016).

Under the initiatives of Department of Biotechnology (DBT), Government of India, and the National Oilseeds and Vegetable Oils Development (NOVOD), a number of *Jatropha* accessions were collected from different locations in India. Their genetic variability, divergence, heritability and genetic advance of the desirable phenotypic traits were studied (Divakara et al., 2010; Tripathi et al., 2013; Dhyani et al., 2015). Thorough survey at different ecogeographic zones in India and other countries only can widen the scope of getting quality germplasm for mass multiplication, field trials, performance evaluation and genetic improvement of *Jatropha*. This report presents an extensive survey for selection and collection of *Jatropha* germplasm from different locations in Punjab, a North-Western state of India, and assessment of the accessions on the basis of genetic variability and divergence in seed traits and oil content.

## 2. Materials and methods

### 2.1. Survey and selection of candidate plus trees (CPTs)

The state of Punjab having subtropical climate is a part of the Indo-Gangetic alluvial plane, composed of sediments of the Siwalik Hills and the Himalayas, having the following physiographic units: Shiwalik hills, Piedmont plain, Alluvial plain, Sand plain, Flood plain and Palaeochannels; which can be further classified into a number of varying agro-climatic zones on the basis of homogeneity, rainfall pattern, soil texture and cropping patterns. A thorough survey was carried out to select a total of 31 morphologically superior *Jatropha* CPTs from different locations of Punjab having latitude, longitude and altitude ranging 30°20′–31°32′N, 74°18′–76°28′E, and 211–260 m, respectively (Table 1). First, the salient morphological attributes of the individual *J. curcas* plantations were recorded, and the CPTs were selected on the basis of some desirable traits (Mishra, 2009). Healthy and mature seeds from each CPT were dried under sun for 1–2 days till they attained constant weight and distributed into five lots. For each seed lot, out of 100 healthy seeds, 10 seeds were chosen randomly to measure length, width and thickness in millimetres, and their averages were recorded; each measurement was replicated thrice. Likewise, 100-seed weight (expressed in grams) was also measured. The *Jatropha* seeds from 31 CPTs were submitted to National Bureau of Plant Genetic Resources (NBPGR), New Delhi with the following Indigenous Collection (IC) numbers: 569342–569358 (except 569345), 561287–561293, 568549–568556 (except 568555), and 560678 (Table 2). The collected *J. curcas* germplasm in terms of both seed- and cutting-raised plants are being maintained in the nursery.

**Table 1**
Ecogeographical characteristics of the survey areas in Punjab for collection of *J. curcas* germplasm.

| District | Location | | | Agro-climatic zones of Punjab |
|---|---|---|---|---|
| | Altitude | Latitude | Longitude | |
| Patiala | 259 m | 30°-20′ N | 76-28′ E | Central plain zone |
| Sangrur | 259 m | 30-20′ N | 76-28′ E | Central plain zone |
| Ludhiana | 259 m | 30-20′ N | 76-28′ E | Central plain zone |
| Barnala | 259 m | 30-20′ N | 76-28′ E | Central plain zone |
| Mansa | 211m | 30-58′ N | 74-18′ E | Western zone |
| Bathinda | 211 m | 30-58′ N | 74-18′ E | Western zone |
| Hoshiarpur | 260 m | 31-32′ N | 75-55′ E | Sub-mountain undulating zone |

### 2.2. Seed oil content

*Jatropha* seed oil% on kernel basis was determined using Soxhlet apparatus (Kumar and Singh, 2014). Briefly, for each seed sample, 50 g kernel was broken into small pieces using mortar and pestle, and kept in the middle chamber of Soxhlet apparatus for oil extraction using hexane as solvent. The extraction process was carried out at 70–80 °C for 8–10 h. During this process, hexane containing the extracted *Jatropha* oil was collected in a round bottom flask for boiling, which allowed hexane to get evaporated but not the oil as the boiling point of the latter was more than hexane. To remove hexane completely from seed oil, the flask was kept in vacuum rotary evaporator. The seed oil% was calculated with the following formula: $[(W_b\text{-}W_a) \times 100]$/kernel weight, where $W_a$ was the weight of empty flask and $W_b$ refers to the weight of flask containing the extracted oil.

### 2.3. Statistical analysis

Analysis of variance (ANOVA) was carried out using the CPCS1 statistical software developed by Punjab Agricultural University, Punjab, India (http://web.pau.edu/). The variability, broad sense heritability, genetic advance as percentage of mean, genotypic coefficient of variation (GCV), phenotypic coefficient of variation (PCV), and linear correlation coefficients for the traits under study were calculated using the methods as suggested by different researchers (Burton, 1952; Johnson et al., 1955; Sendecor and Cochran, 1967). Non-hierarchical (K-means) Euclidean cluster analysis was carried out using IBM SPSS Statistics 21 software to assess the extent of broad genetic divergence (Sachan et al., 2004).

## 3. Results

### 3.1. Variability in seeds

The seeds from the *J. curcas* CPTs showed considerable variation with regard to shape, size and color of the seed coats (Fig. 1). As evident from ANOVA, significant differences ($P < 0.05$) were noticed in the seed characteristics between the accessions (Table 2). TJS–46#04, TJS–27#108 and TJS–06#24 were found to be comparable in terms of maximum seed length (i.e., 18.49 mm, 18.48 mm and 18.47 mm, respectively). Minimum seed length was noticed in TJS–15#11 (16.18 mm) followed by TJS–02#01 (16.60 mm). Seed width ranged from 10.65 mm to 11.82 mm. TJS–33#14 showed maximum seed width (11.82 mm) followed by TJS–46#04 (11.69 mm); minimum seed width was noticed in TJS–42#01 (10.65 mm). The seed thickness varied from TJS–19#01 (7.89 mm) to TJS–39#73 (8.85 mm).

### 3.2. Seed weight and oil content

The CPTs showed significant variation in terms of both 100-seed weight and oil content (Table 2). The range of 100-seed weight varied markedly from 35.10 g (TJS–02#01) to 77.34 g (TJS–31#13). For majority of the accessions, the values were found to be in the range of 60–70 g; however, there were 6 accessions which showed the values above this range. With regard to seed oil content, variation was also significant between the accessions. For this trait, the values were between 13.74% (TJS–02#01) and 54.37% (TJS–04#42). 24 accessions showed 30–40% oil content; 3 accessions showed more than 40% oil content, and for the remaining it was less than 30%.

**Table 2**
Seed traits and oil content variability in *J. curcas* accessions collected in the state of Punjab, India.

| CPT | Acc. No. | NBPGR IC No. | Seed length (mm) | Seed width (mm) | Seed thickness (mm) | 100 seed weight (g) | Oil content (%) |
|---|---|---|---|---|---|---|---|
| 1 | TJS 01#04 | IC-568549 | 17.14 | 10.69 | 8.07 | 65.58 | 37.35 |
| 2 | TJS 02#01 | IC-569343 | 16.60 | 10.66 | 8.17 | 35.10 | 13.74 |
| 3 | TJS 04#42 | IC-561287 | 17.23 | 11.28 | 8.39 | 69.86 | 54.37 |
| 4 | TJS 05#10 | IC-561288 | 17.04 | 11.05 | 7.89 | 66.65 | 42.05 |
| 5 | TJS 06#24 | IC-561289 | 18.47 | 11.56 | 8.45 | 69.95 | 33.16 |
| 6 | TJS 07#05 | IC-569342 | 17.92 | 10.69 | 8.55 | 74.07 | 29.85 |
| 7 | TJS 15#11 | IC-569344 | 16.18 | 10.97 | 8.07 | 43.36 | 18.24 |
| 8 | TJS 17#01 | IC-561290 | 17.73 | 10.88 | 8.48 | 63.73 | 32.59 |
| 9 | TJS 19#01 | IC-568551 | 17.48 | 10.78 | 7.89 | 65.73 | 38.41 |
| 10 | TJS 21#01 | IC-568550 | 17.41 | 11.14 | 8.59 | 60.26 | 33.58 |
| 11 | TJS 23#17 | IC-569346 | 18.29 | 11.48 | 8.22 | 66.50 | 45.17 |
| 12 | TJS 25#01 | IC-568552 | 17.24 | 11.10 | 8.47 | 60.65 | 38.86 |
| 13 | TJS 26#39 | IC-561292 | 17.87 | 11.01 | 8.37 | 60.90 | 35.65 |
| 14 | TJS 27#108 | IC-568554 | 18.48 | 11.21 | 8.44 | 69.58 | 32.93 |
| 15 | TJS 28#01 | IC-568556 | 17.32 | 11.28 | 8.18 | 67.86 | 35.52 |
| 16 | TJS 29#07 | IC-569347 | 17.81 | 11.51 | 8.15 | 73.99 | 35.44 |
| 17 | TJS 30#06 | IC-561291 | 17.66 | 11.42 | 8.54 | 71.65 | 35.45 |
| 18 | TJS 31#13 | IC-569348 | 17.96 | 10.91 | 8.36 | 77.34 | 36.04 |
| 19 | TJS 32#16 | IC-569349 | 17.92 | 11.38 | 8.36 | 68.35 | 35.51 |
| 20 | TJS 33#14 | IC-569350 | 17.45 | 11.82 | 8.04 | 62.94 | 33.28 |
| 21 | TJS 34#37 | IC-569351 | 17.20 | 11.06 | 8.67 | 59.55 | 34.98 |
| 22 | TJS 35#01 | IC-569352 | 17.11 | 11.31 | 8.55 | 58.06 | 35.20 |
| 23 | TJS 36#23 | IC-561293 | 17.17 | 11.27 | 8.50 | 71.33 | 36.12 |
| 24 | TJS 37#33 | IC-560678 | 17.91 | 11.38 | 8.72 | 69.01 | 33.95 |
| 25 | TJS 38#03 | IC-568553 | 17.72 | 11.42 | 8.52 | 62.59 | 36.54 |
| 26 | TJS 39#73 | IC-569353 | 17.30 | 11.42 | 8.85 | 70.61 | 28.56 |
| 27 | TJS 40#05 | IC-569354 | 18.35 | 11.38 | 8.70 | 64.17 | 36.94 |
| 28 | TJS 41#14 | IC-569355 | 17.66 | 11.30 | 8.73 | 68.23 | 31.54 |
| 29 | TJS 42#01 | IC-569356 | 16.74 | 10.65 | 8.15 | 50.33 | 39.64 |
| 30 | TJS 46#04 | IC-569357 | 18.49 | 11.69 | 8.77 | 60.79 | 39.58 |
| 31 | TJS 49#01 | IC-569358 | 17.65 | 11.22 | 8.61 | 64.70 | 32.20 |
| SEm( ± ) | | | 0.11 | 0.51 | 0.62 | 0.17 | 1.54 |
| | CD at 5% | | 0.41 | 0.28 | 0.31 | 0.67 | 2.02 |

### 3.3. Genetic variability studies in seed traits

The extent of genetic variability expressed in different seed and oil traits was examined by the studies that included genotypic and phenotypic variance, GCV, PCV along with broad sense heritability and genetic advance. The magnitude of variability corresponding to some seed traits of *J. curcas* are shown in Table 3. For the traits namely length, width and thickness, the magnitude of both phenotypic variance and PCV were found to be higher in comparison to the corresponding genotypic variance and GCV. The extent of both genotypic and phenotypic variance was highest and comparable for 100-seed weight (i.e., around 76) followed by nearly comparable for seed oil content (i.e., around 49). Similar pattern was noticed for GCV and PCV; the values for oil content were highest and comparable (i.e., around 20) followed by 100-seed weight (i.e., around 14). The highest broad sense heritability of 99% was recorded for 100-seed weight followed by oil content (97%) and seed length (81%); whereas seed thickness showed the lowest heritability of 17% followed by 32% as found in seed width. The genetic advance as represented by percent of mean ranged from 0.30% for seed thickness to 17.83% for 100-seed weight. Correlation coefficient between seed and oil traits are presented in Table 4. Positive and significant correlation was observed between 100-seed weight and oil content (r = 0.517). Likewise, seed length and 100-seed weight were positively and significantly correlated. Seed length showed positive correlation with oil content and seed width. Seed width showed positive correlation with all other traits under study. Seed thickness was positively correlated with other traits except oil content as it showed negative and insignificant correlation.

### 3.4. Divergence analysis

Based on the non-hierarchical *K*-Means cluster analysis, the CPTs were grouped into 6 clusters (Table 5). 12 accessions were included in cluster VI followed by cluster I (9 accessions) and cluster IV (6 accessions) and cluster II (2 accessions), whereas both the clusters III and V included only 1 accession. The inter-cluster distances are presented in Table 6. The highest inter-cluster distance i.e., 49.114 was found between the clusters II and III followed by the value 36.389 between the clusters II and VI. The minimum inter-cluster distance i.e., 6.194 was noted between cluster I and IV. Cluster mean values for seed and oil traits are shown in Table 7.

## 4. Discussion

Currently, large-scale cultivation of superior *Jatropha* varieties is being promoted in many countries because they can serve as potential renewable substitute for fossil fuels. Seed yield and oil content are regarded as two major traits for any biodiesel crop. To realize the full potential of this biodiesel crop, we still need extensive survey, selection and collection of both local and global *Jatropha* germplasm from different eco-geographic locations followed by assessment of genetic variability and diversity for various desirable traits. Apart from annual seed yield per plant, an important attribute of the elite *Jatropha* accessions includes 30–40% oil content with the presence of high amounts of mono-unsaturated fatty acids such as C16:1 and C18:1 (Knothe, 2009). During survey, the salient morphological attributes of the individual *J. curcas* plantations were first recorded; the CPTs were selected on the basis of some desirable traits such as seed yield (2–5 kg/plant), number of mature fruits per raceme, branching patterns with compact canopy, height, crown spread, collar diameter, disease resistance, plant hardiness, and synchronized maturity. Although each of these attributes is important, some criteria are considered preferentially over others during systematic evaluation and selection of a CPT (Mishra, 2009).

Germplasm in the form of seeds for any tree species are known to vary considerably with regard to morphological and physiological


**Fig. 1.** Harvested mature seeds from different *J. curcas* accessions: TJS-01#04 (a); TJS-04#42 (b); TJS-05#10 (c); TJS-07#05 (d); TJS-15#11 (e); TJS-23#17 (f); TJS-27#108 (g); TJS-29#07 (h); TJS-36#23 (i).

**Table 3**
Determination of genotypic and phenotypic variables for seed and oil traits in *J. curcas*.

| Traits | Variance | | Coefficient of variation (%) | | Heritability (broad sense) | Genetic advance as % of mean |
|---|---|---|---|---|---|---|
| | Genotypic | Phenotypic | Genotypic | Phenotypic | | |
| Seed length | 0.48 | 0.59 | 3.93 | 4.38 | 81 | 1.28 |
| Seed width | 0.24 | 0.75 | 4.39 | 7.70 | 32 | 0.57 |
| Seed thickness | 0.13 | 0.75 | 4.28 | 10.36 | 17 | 0.30 |
| 100-seed weight | 75.91 | 76.08 | 13.55 | 13.56 | 99 | 17.83 |
| Oil content | 47.91 | 49.45 | 19.82 | 20.13 | 97 | 14.04 |

parameters depending on the prevailing eco-geographic conditions. Such variations are due to both genotype as well as their adaptation to different environmental conditions (Mathur et al., 1984; Senger et al., 2016). Soil properties and climatic conditions influence significantly *J. curcas* productivity. Several factors like sandy-loam soil, tropical/sub-tropical climate, and adequate annual precipitation are suitable for overall growth and high seed yield. *J. curcas* shows poor growth and low productivity in the arid and semi-arid climates with poor soil nutrients (Maes et al., 2009; Trabucco et al., 2010; Valdes-Rodriguez et al., 2011). The CPTs under study are adaptive to different agro-climatic zones of Punjab that include wide range of rainfall, temperature and soil types. Most of the *J. curcas* CPTs were selected from the plantations located in the central plain zone having sandy-loam soil with annual rainfall ranging from 600 to 900 mm. A number of accessions appeared to be promising in terms of growth, high seed yield and oil content. A few accessions namely TJS–37#33, TJS–42#01 and TJS–49#01 corresponding to sub-mountain undulating zone with sandy-loam soil having a higher rainfall (800–1500 mm) also showed similar desirable traits (Tables 1 and 2). All these observations are consistent with the earlier reports. Due to wide distribution range of *J. curcas*, there is a scope to identify genetic variation in the germplasm. The analyses of seed and oil traits of the mature seeds revealed

**Table 4**
Phenotypic correlation coefficient between seed and oil traits in *J. curcas*.

| Traits | 100-seed weight | Oil content | Seed length | Seed width |
|---|---|---|---|---|
| Oil content | 0.517 | | | |
| Seed length | 0.620 | 0.312 | | |
| Seed width | 0.336 | 0.246 | 0.479 | |
| Seed thickness | 0.226 | −0.037 | 0.402 | 0.337 |

Correlation is significant at 5% level.

**Table 5**
Non-hierarchical K-Means clusters for seed and oil traits in *J. curcas* accessions.

| Cluster | Number of accessions | Accession names[a] |
|---|---|---|
| I | 9 | TJS-17#01 (2.725), TJS-21#01 (1.903), TJS-26#39 (1.519), TJS-33#14 (1.819), TJS-34#37 (2.415), TJS-35#01 (3.905), TJS-38#03 (2.122), TJS-40#05 (3.401), TJS-49#01 (3.675) |
| II | 2 | TJS-02#01 (4.711), TJS-15#11 (4.711) |
| III | 1 | TJS-04#42 (0.000) |
| IV | 6 | TJS-01#04 (3.220), TJS-05#10 (3.029), TJS-19#01 (2.363), TJS-23#17 (5.448), TJS-25#01 (3.943), TJS-46#04 (3.775) |
| V | 1 | TJS-42#01 (0.000) |
| VI | 12 | TJS-06#24 (1.376), TJS-07#05 (4.941), TJS-27#108 (1.741), TJS-28#01 (3.685), TJS-29#07 (3.499), TJS-30#06 (1.905), TJS-31#13 (6.783), TJS-32#16 (3.229), TJS-36#23 (2.549), TJS-37#33 (2.026), TJS-39#73 (5.166), TJS-41#14 (3.505) |

[a] Euclidean distance for each cluster member is indicated within the parenthesis.

**Table 6**
Inter-cluster distances based on seed and oil traits in *J. curcas* accessions.

| Cluster | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| I | – | 29.313 | 21.370 | 6.194 | 12.668 | 9.164 |
| II | | – | 49.114 | 34.912 | 26.128 | 36.389 |
| III | | | – | 15.188 | 24.476 | 20.737 |
| IV | | | | – | 14.035 | 9.373 |
| V | | | | | – | 21.550 |
| VI | | | | | | – |

**Table 7**
Cluster mean value for seed and oil traits in *J. curcas* accessions.

| Cluster | Seed length | Seed breadth | Seed thickness | Seed weight | Oil content |
|---|---|---|---|---|---|
| I | 17.61 | 11.25 | 8.50 | 61.88 | 34.55 |
| II | 16.39 | 10.82 | 8.12 | 39.23 | 15.99 |
| III | 17.23 | 11.28 | 8.39 | 69.86 | 54.37 |
| IV | 17.61 | 11.13 | 8.22 | 64.32 | 40.24 |
| V | 16.74 | 10.65 | 8.15 | 50.33 | 39.64 |
| VI | 17.80 | 11.28 | 8.49 | 71.00 | 33.67 |

considerable variation between the CPTs. The extent of variability was analyzed by some useful parameters such as genotypic and phenotypic variances (GV and PV) along with genotypic and phenotypic coefficient of variation (GCV and PCV). As revealed in the earlier reports, the GCV was found to be lower than PCV for all the seed and oil traits namely 100-seed weight, seed:kernel (S/K) ratio, oil content, seed length, seed width and seed thickness which suggests that the characteristics were least influenced by the environment (Ginwal et al., 2004; Kaushik et al., 2007; Gohil and Pandya, 2008).

Heritability is an important aspect in the areas of conventional breeding and genetics as it provides an estimate of the role of genetic factors that influence variation in the phenotypic traits. Nearly 100% heritability was noticed for number of fruits per plant, and it was 88.79% for number of inflorescence per plant in *J. curcas* (Mohapatra and Panda, 2010). Likewise, high heritability and genetic advance were reported for the following traits: fruit yield per plant ($H^2$ = 92.22 and GA = 76.82) and seed yield per plant ($H^2$ = 88.67 and GA = 56.66) (Tripathi et al., 2013). Heritability values with genetic advance were duly considered for screening and selection of the CPTs under study. High broad sense heritability with moderate genetic advance was observed in the cases of 100-seed weight ($H^2$ = 99.00 and GA = 17.83) and oil content ($H^2$ = 97.00 and GA = 14.04). These traits need to be considered for direct selection of the elite *J. curcas* germplasm. Seed length ($H^2$ = 81.00 and GA = 1.28) showed high heritability but with lower genetic advance. This could be due to the presence of non-additive gene effects and higher genotypic and environmental interactions. The traits like seed width and seed thickness recorded low heritability and low genetic advance indicating the predominance of non-additive gene interactions. As reported earlier, significant positive correlation was found between seed oil content and 100-seed weight suggesting the effectiveness of indirect selection on the basis of these traits (Rao et al., 2008; Tripathi et al., 2013). However, there was little positive correlation between 100-seed weight and oil content (r = 0.235) (Halilu et al., 2011). In this study, positive and significant correlation was noticed between the traits like 100-seed weight and oil content (r = 0.517). The trait like seed length was found to be positively correlated with 100-seed weight, oil content, seed thickness and seed width. It is likely that the traits showing considerable correlation are controlled by the genes which are closely linked.

As evident from the *K*-Means cluster analysis, there was no direct correlation between agro-climatic diversity and genetic diversity. Wider genetic diversity was noticed between the clusters II and III followed by the clusters II and VI. Significant variation was noticed between the clusters with regard to oil content and seed weight. Cluster III with a single member showed maximum oil content and higher values for the seed traits like seed weight, length, breadth and thickness. In addition to Cluster III, both the clusters IV and VI showed considerably higher values for the oil content and the other seed traits. Particularly, the CPTs of the clusters III, IV and VI could be used for selection and further tree improvement programmes.

## 5. Conclusions

Extensive survey and analysis of the seed and oil traits helped to identify a number of superior *J. curcas* genotypes which could be used for large-scale plantations with proper agronomic practices. Some of the *J. curcas* CPTs of this study are quite promising and can serve the purpose of germplasm exchange, mass propagation, evaluation of their field performance through multi-location trials, micropropagation for generation of true-to-type propagules, genetic improvement through conventional/marker-assisted breeding, somaclonal variants, mutant population, doubled haploids, inter-specific hybridization and transgenics for the desired phenotypic traits such as increasing seed yield and oil content per unit area on a sustainable basis. Apart from specific breeding purposes, the selected *J. curcas* genotypes showing more than 30% oil content will be useful for cultivation to improve seed yield, and to maximize oil extraction process for both fatty acid profile analysis and biodiesel production.

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# Seed oil of *Jatropha curcas* L. germplasm: Analysis of oil quality and fatty acid composition

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ABSTRACT

*Jatropha curcas* L. is recognized as one of the important non-edible tree borne oilseeds (TBOs) in India and many other tropical countries. *J. curcas* is a promising bioenergy crop as its seed oil is a suitable feedstock for biodiesel production. This study focused on a total of 19 *J. curcas* accessions for seed oil extraction, oil quality analysis and fatty acid composition. Most of the accessions showed more than 30% oil content with free fatty acid content ranging from 0.21 to 1.82%. The oil samples were transesterified efficiently to fatty acid methyl esters as evident from proton nuclear magnetic resonance ($^{1}$H NMR) spectra. As revealed by gas chromatography, the contents of the four major fatty acids were found to significantly vary in the seed oils viz Palmitic acid (8.64–17.05%), stearic acid (4.34–7.94%), oleic acid (26.26–46.36%) and linoleic acid (28.72–53.78%). A number of seed oils showed high level of oleic acid (40.02–46.36%), and some other oil samples were rich in linoleic acid (~45%). These *J. curcas* accessions appeared to be promising with regard to clonal propagation, field trials, large-scale plantations for biodiesel feedstocks, other industrial applications, and also as prebreeding materials in crop improvement programs.

## 1. Introduction

Currently, many countries in the world focus on alternative energy sources to overcome the major challenges due to fast-depleting petroleum reserves and adverse effects of the greenhouse gas emissions (Balat and Balat, 2010; Huerga et al., 2014). Biological sources particularly fat- and oil-derived fuels are gaining considerable importance. Raw vegetable oil (RVO) is not applied as a fuel in CI engines because of very poor atomization, high viscosity, low volatility, and partial combustion causing increased soot and smoke formation (Nayak et al., 2017). The term 'biodiesel' refers to mono-alkyl esters of fatty acids having the following desirable attributes: renewability, biodegradability, low sulphur content, good lubricity, higher flash point and ease of oil extraction and processing. It can be blended in varying proportions with non-renewable petro-fuels. In addition, combustion of biodiesel produces reduced level of particulate matters, carbon monoxide (CO), hydrocarbons, soot and sulphur oxides (SOx). Relatively a new term i.e., 'renewable diesel' is coined which resembles petrodiesel produced by cracking or pyrolysis; hydrodeoxygenation is also gaining importance in the recent years (Berchmans and Hirata, 2008; Fukuda et al., 2001; Knothe, 2009, 2010).

USA and many countries in Europe and South-East Asia use various plant edible oils such as soybean, rapeseed, peanut, sunflower and palm seeds for biodiesel production. There is a short-supply of edible oils in Indian subcontinent; therefore, the focus has been shifted to various tree-borne oilseeds (TBOs) such as Neem (*Azadirachta indica*), Karanj (*Pongamia pinnata*), Mahua (*Madhuca indica*), Jatropha (*Jatropha curcas*) because a) they grow under different agro-climatic and edaphic conditions, b) ease of large-scale production of raw materials i.e., oilseeds, oil extraction, and process of transesterification, c) supportive to sustainable development, energy conservation, environment preservation, rural development, poverty alleviation and waste land reclamation (Achten et al., 2008; Demirbus, 2008; Dhyani et al., 2015). *J. curcas* also known as physic nut, a member of the *Euphorbiaceae* family, is a fast-growing, deciduous non-edible oilseed plant and shows considerable tolerance to drought and pest. It is a perennial multipurpose tree and gaining importance increasingly as a promising bioenergy crop because its seed oil, in particular, serves as a suitable feedstock in the biodiesel industries. As evident in the earlier reports, seed yield and oil content vary significantly between the *J. curcas* accessions. These traits are influenced by genotype, eco-geographic conditions, agronomic practices and various stresses (Divakara et al., 2010; Srivastava et al., 2011; Quinn et al., 2015).

The production of biodiesel from *J. curcas* seed oil through transesterification has become an area of intense research in the recent years. During this process, triacylglycerol (TAG) molecules react with an alcohol namely methanol or ethanol in the presence of a catalyst,

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usually potassium hydroxide to form fatty acid methyl/ethyl esters and glycerol. Various parameters are known to influence the tranesterification reaction involving vegetable oils as evident from several reports. For example, oil resources and its fatty acid composition, oil quality, nature of the contaminants and their contents, methanol-to-oil ratio, temperature, nature of catalysts, reactor/separator design, hydrodynamic conditions such as volumetric flow rates, phase ratios and catalyst concentrations and other process conditions influence the transesterfication process. Some lipases are being used as substitute of base catalysts in the transesterification process (Ghesti et al., 2007; Klofutar et al., 2010; Wang et al., 2011; Likozar and Levec, 2014; Likozar et al., 2016; Rodrigues et al., 2016).

Quantitatively, seed oil content is a desirable attribute of the individual *J. curcas* accessions; but the parameters like free fatty acid (FFA) content and fatty acid composition (FAC) are major influencing factors in determining the quality of a biodiesel (Tiwari et al., 2007; Wang et al., 2011). High FFA content has adverse effects on the transesterification process because of soap formation causing low yield of biodiesel product. FAC analysis helps to assess the fuel properties of a biodiesel such as cetane number (CN), oxidative stability (OS), viscosity, lubricity and cold flow properties (Demirbus, 2008; Knothe, 2009; Ramos et al., 2009). The composition of a quality biodiesel should comply with the following parameters: presence of high amounts of monounsaturated fatty acids (MUFA) such as C16:1 and C18:1, reduced level of the polyunsaturated fatty acids (PUFA) and optimal presence of the saturated fatty acids (Knothe, 2009; Pinzi et al., 2009). Apart from searching the superior *J. curcas* genotypes with high seed oil content and desired FAC, some other strategies were also adopted successfully in developing the designer crops. For example, Qu et al. (2012) reported marker-free RNA interference transgenic *J. curcas* plants with significantly increased level of oleic acid in seed oil. A few *J. curcas* lines with very high oleic acid content were generated through genetic crossing (Sinha et al., 2016).

A thorough survey and analysis of the seed and oil traits helped us to identify a number of superior *J. curcas* genotypes in terms of both seed yield and oil content from different locations in Punjab, a North-Western state of India (Kumar and Das, 2018). This report presents extraction of seed oil from the *J. curcas* accessions, analysis of oil and FFA content, transesterification, quality checking of the fatty acid methyl esters by $^1$H NMR followed by FAC analysis by gas chromatography (GC). The objective was to identify the promising *J. curcas* accessions suitable for multi-location trials, performance evaluation, large-scale cultivation, biodiesel production and crop improvement.

## 2. Materials and methods

### 2.1. Plant materials and reagents

A number of morphologically superior *J. curcas* candidate plus trees (CPTs) were identified from different agro-climatic zones covering some districts of Punjab, a North-Western state of India. Ecogeographical characteristics of the survey and collection sites are provided in Table 1. This study focused on a total of 19 *J. curcas* accessions. The mature seeds collected from these accessions were submitted to National Bureau of Plant Genetic Resources (NBPGR), New Delhi for assignment of the Indigenous Collection (IC) numbers. Methanol, potassium hydroxide, hexane and other chemicals were of analytical grade, and purchased from Himedia. For GC analysis, a standard fatty acid methyl ester (FAME) $C_8$–$C_{24}$ was purchased from Sigma-Aldrich.

### 2.2. Extraction of seed oil and analysis of FFA content

*J. curcas* seed oil % on kernel basis was determined using Soxhlet apparatus (Kumar and Singh, 2014). Briefly, the healthy *Jatropha* seeds were dried under sun, followed by drying in hot air oven at 40 °C till they attain constant weight. For each seed sample, 50 g of kernel was broken into small pieces, and oil extraction was carried out at 70–80 °C for 8–10 h using hexane as solvent. To remove hexane completely from seed oil, the flask was kept in vacuum rotary evaporator. The oil % was calculated using the following equation

$$\% \text{ Oil} = [(W_b - W_a) \times 100]/\text{kernel weight} \quad (1)$$

where $W_a$ was the weight of empty flask and $W_b$ refers to weight of flask containing the extracted oil. The free fatty acid (FFA) content of each seed oil sample was determined by the titrimetric method (Rukunudin et al., 1998). Briefly, 10 g of oil was dissolved in a mixture of ethanol and diethyl ether (1:1 volume ratio), and titrated with 0.1 M KOH solution using phenolphthalein as indicator. FFA concentration in seed oil was calculated as percentage oleic acid on the basis of the following equation

$$\% \text{ FFA as Oleic acid} = [\text{alkali volume (mL)} \times \text{alkali normality} \times 28.2]/\text{Sample weight (g)} \quad (2)$$

**Table 1**
Ecogeographical characteristics of the survey areas of the North-Western part of India for collection of the *J. curcas* accessions.

| S. No. | TI Acc. No. | District | Location | | | Agro-climatic zones of Punjab |
|---|---|---|---|---|---|---|
| | | | Altitude | Latitude | Longitude | |
| 1 | TJS-01# 04 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 2 | TJS-02#01 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 3 | TJS-04#42 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 4 | TJS-05#10 | Mansa | 211 m | 30°–58' N | 74°–18' E | Western zone |
| 5 | TJS-06#24 | Sangrur | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 6 | TJS-07#05 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 7 | TJS-15#11 | Hoshiarpur | 260 m | 31°–32' N | 75°–55' E | Sub-mountain undulating zone |
| 8 | TJS-17#01 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 9 | TJS-19#01 | Ludhiana | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 10 | TJS-21#01 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 11 | TJS-23#17 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 12 | TJS-25#01 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 13 | TJS-27#108 | Ludhiana | 259 m | 30°–20' N | 76°-28' E | Central plain zone |
| 14 | TJS-28#01 | Patiala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 15 | TJS-29#07 | Barnala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 16 | TJS-30#06 | Barnala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 17 | TJS-31#13 | Barnala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 18 | TJS-32#16 | Barnala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |
| 19 | TJS-33#14 | Barnala | 259 m | 30°–20' N | 76°–28' E | Central plain zone |

### 2.3. Transesterification

The extracted *J. curcas* seed oil samples were treated with methanol in presence of KOH to produce fatty acid methyl esters. Briefly, 10 g of each seed oil was mixed with 2.2 mL methanol (1:6 oil-to-alcohol mole ratio) and 0.1 g KOH (1% w/w of oil) in 50 mL beaker. The reaction mixture was kept on hot air magnetic stirrer at 70 °C for 2 h with continuous stirring using a magnetic bead. The mixture was then shifted to a separating funnel and kept undisturbed overnight to allow complete separation of two layers in the form of methyl esters and glycerol. After removal of glycerol, the remaining product was washed 2–3 times with aqueous solution of HCl (0.5 wt %) and NaCl (5.0 wt %), and dried over anhydrous magnesium sulphate, and residual methanol was removed in a rotary evaporator at 70 °C (Ghesti et al., 2007). Proton nuclear magnetic resonance ($^1$H NMR) method was employed to assess the quality, particularly with regard to FFA content, of the biodiesel i.e., fatty acid methyl ester as-synthesized (Satyarthi et al., 2009). For $^1$H NMR, 15–20 mg of the sample was dissolved in 0.6 mL of $CDCl_3$; the spectrum was recorded at 298 K on a Jeol ECS 400 MHz spectrometer (during the measurement, 64 scans were taken for each sample). A standard 5 mm quadronuclei ($^1$H, $^{31}$P, $^{13}$C, and $^{15}$N) probe (QNP) was used. The other related parameters such as acquisition time of 3.9 s, relaxation delay of 1 s, flip angle of 45°, and sweep width of 4.139 kHz were employed during the spectral measurements.

### 2.4. Fatty acid composition by gas chromatography

The individual fatty acid methyl esters were used for quantitative analysis of fatty acid composition by gas chromatography (GC). An Agilent EC-5GC instrument equipped with flame ionization detector, automatic sample injector along with a separation column namely SP-2560 (30.0 m × 0.53 mm × 1.2 µm) was used. The other conditions were: inlet temperature (150 °C), detector temperature (260 °C), split ratio (19:1), oven temperature programme (140 °C–240 °C at 4 °C/min), hold for 15 min, injection volume (1.0 µL); carrier gas: $N_2$ at 2 mL/min; air flow: 450 mL/min; $H_2$ flow: 45 mL/min. Under these conditions, GC chromatograms of the standards (procured from Sigma-Aldrich, USA) and samples were obtained with an injection volume of 1.0 µL. The chromatographic run time for each sample was around 60 min.

### 2.5. Statistical analysis

The descriptive statistical data such as mean, standard deviation (SD), variance and range were generated on the basis of fatty acid composition of the seed oil samples from the *J. curcas* accessions. SPSS software (Statistical Product and Service Solutions) Version 17 (IBM, New York, USA) was employed in statistical analysis.

## 3. Results and discussion

### 3.1. Seed oil and FFA content analysis

Seed oil content, FFA content and FAC of the *J. curcas* accessions were analysed as shown in Table 2. Oil content was found to vary from 13.74% (TJS-02#01) to 54.37% (TJS-04#42). Most of them showed 30–40% oil content, and the value was found to be more than 40% in a few accessions. Seed oil content is one of the important parameters that need to be considered during selection of a candidate plus trees for any TBO. Seed stocks of most *J. curcas* accessions under study were acceptable in terms of their contribution to overall yield of raw vegetable oil by the extraction process as described earlier. FFA content was determined by titrimetric method and the values ranged from 0.21 to 1.82% in most of the oil samples except one accession i.e., TJS-02#01 (4.81%). 14 out of 19 oil samples of the study showed less than 1.0% FFA content which is consistent with the earlier reports. Aminul Islam et al. (2012) analyzed *J. curcas* seed oil from 24 candidate plus plants (CPPs) of 9 geographical origins. The seed oil content varied from 32.1% to 48.8%; the values of FFA content ranged from 0.3 to 2.3%, and it was less than 1% for most of the CPPs. Kaushik and Bhardwaj (2013) collected *J. curcas* seeds from 14 different geographical locations in India; the oil content was found to vary significantly i.e., 11.68%–42.08% depending on location and soil type.

As evident from several reports, various biometric and biochemical traits along with seasonal, agronomic and genetic factors influence seed yield, oil content, FAC, FFA concentration and other attributes in *J. curcas* (Kaushik and Bhardwaj, 2013; Sinha et al., 2015). $^1$H NMR spectra revealed that triacylglycerol (TAG) level was significantly low as compared to FFA at early developing stages of *J. curcas* seeds. There was a sharp decline of FFA level at the maturation stages as they are replaced by TAG, the predominant lipid in seeds (Annarao et al., 2008).

**Table 2**
Oil content (%, w/w), FFA content (%, w/w), and fatty acid composition (%, w/w) of seed kernels from the selected *J. curcas* accessions.

| S. No. | TI Acc. No. | NBPGR IC No. | Oil content | FFA content | Palmitic acid | Stearic acid | Oleic acid | Linoleic acid | Total UFA |
|---|---|---|---|---|---|---|---|---|---|
| 1. | TJS-01#04 | IC-568549 | 37.35 | 1.22 | 12.44 | 7.94 | 46.36 | 28.72 | 75.08 |
| 2. | TJS-02#01 | IC-569343 | 13.74 | 4.81 | 12.65 | 6.38 | 33.22 | 45.90 | 79.12 |
| 3. | TJS-04#42 | IC-561287 | 54.37 | 0.48 | 14.44 | 5.66 | 38.36 | 40.54 | 78.90 |
| 4. | TJS-05#10 | IC-561288 | 42.05 | 0.99 | 17.05 | 4.39 | 30.57 | 46.25 | 76.82 |
| 5. | TJS-06#24 | IC-561289 | 33.16 | 0.34 | 11.49 | 6.53 | 41.57 | 39.89 | 81.46 |
| 6. | TJS-07#05 | IC-569342 | 29.85 | 0.24 | 11.03 | 5.44 | 38.52 | 44.26 | 82.78 |
| 7. | TJS-15#11 | IC-569344 | 18.24 | 1.82 | 10.77 | 6.06 | 42.84 | 40.98 | 83.82 |
| 8. | TJS-17#01 | IC-561290 | 32.59 | 0.93 | 13.28 | 5.12 | 41.93 | 38.68 | 80.01 |
| 9. | TJS-19#01 | IC-568351 | 38.41 | 1.04 | 10.71 | 6.87 | 44.17 | 36.68 | 80.85 |
| 10. | TJS-21#01 | IC-568550 | 33.58 | 0.40 | 11.74 | 5.68 | 32.16 | 48.95 | 81.11 |
| 11. | TJS-23#17 | IC-569346 | 45.17 | 0.62 | 15.70 | 6.00 | 40.02 | 35.28 | 75.30 |
| 12. | TJS-25#01 | IC-568552 | 38.86 | 0.87 | 10.39 | 5.80 | 45.86 | 34.84 | 80.70 |
| 13. | TJS-27#108 | IC-568554 | 32.93 | 1.00 | 10.72 | 5.86 | 42.25 | 40.50 | 82.75 |
| 14. | TJS-28#01 | IC-568556 | 35.52 | 0.37 | 11.96 | 4.34 | 32.60 | 50.02 | 82.62 |
| 15. | TJS-29#07 | IC-569347 | 35.44 | 0.78 | 12.78 | 6.08 | 38.60 | 41.38 | 79.98 |
| 16. | TJS-30#06 | IC-561291 | 35.45 | 0.37 | 13.21 | 4.36 | 38.98 | 42.02 | 81.00 |
| 17. | TJS-31#13 | IC-569348 | 36.04 | 0.33 | 8.64 | 5.89 | 44.18 | 40.72 | 84.90 |
| 18. | TJS-32#16 | IC-569349 | 35.51 | 0.22 | 11.94 | 5.60 | 32.20 | 48.50 | 80.70 |
| 19. | TJS-33#14 | IC-569350 | 33.28 | 0.21 | 14.35 | 4.79 | 26.26 | 53.78 | 80.04 |

*FFA* free fatty acid; *UFA* unsaturated fatty acid.
Values are the means of triplicate assay.

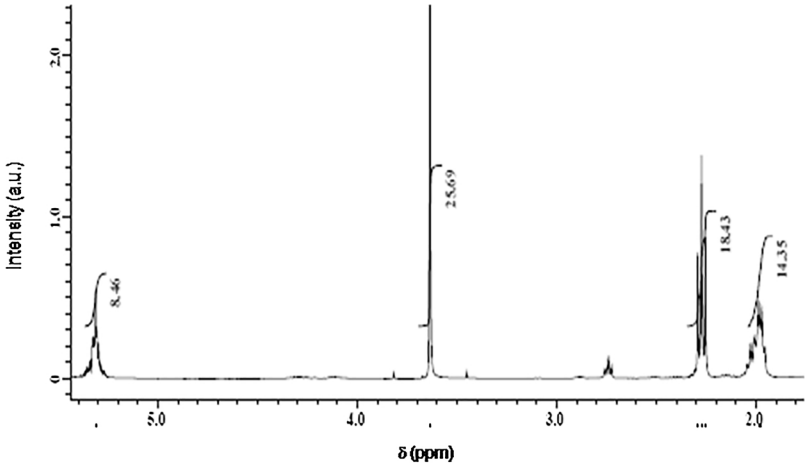


**Fig. 1.** $^{1}$H NMR spectra of fatty acid methyl ester (biodiesel) corresponding to the seed oil of *J. curcas* accession (TJS-01#04).

In plants, diacylglycerol *O*-acyltransferase (DGAT) is committed to TAG biosynthesis, and involved in plastidial fatty acid flux control in oil biosynthesis. There was a significant increase of oil content in both seeds and leaves in the transgenic *J. curcas* lines showing constitutive over expression of *AtDGAT1,* and extracted crude oil showed reduced FFA level in comparison to control wild-type plants (Maravi et al., 2016). It is likely that a multitude of factors including stresses may delay or interfere in the synthesis of fatty acids, TAG and other downstream pathways contributing to variation in FFA level in seeds of the chosen *J. curcas* accessions.

Apart from aforesaid metabolic flux control during seed development, other parameters like extraction process, improper handling, inappropriate storage condition, duration of storage, action of lipases may contribute to higher FFA level in seed oil. FFA content is recognized as one of the important physico-chemical properties with regard to quality of a vegetable oil. The value less than 1% is suitable for conversion to methyl esters; exceeding more than 2 wt % significantly compromised both the yield and recovery of biodiesel due to saponification during the process of base-catalyzed transesterification. High FFA content in *J. curcas* oil could be overcome by the two-step conversion process i.e., lowering FFA content by an acid-catalyzed esterification pretreatment prior to base-catalyzed transesterification (Knothe, 2005; Tiwari et al., 2007; Berchmans and Hirata, 2008; Chai et al., 2014). Most of *J. curcas* seed oil samples of the study were found suitable in terms of low FFA content and efficient transesterification.

### 3.2. Transesterification and FAC analysis

The individual seed oil samples were used to carry out transesterification for biodiesel production. The quality of the resulting fatty acid methyl esters was assessed by $^{1}$H NMR analysis. This technique is also known to be useful for detection of the presence FFA in the oil samples (Satyarthi et al., 2009). The distinct peak of the biodiesel ($\delta$ value ~3.67) corresponding to the *J. curcas* accession TJS-01#04 seed oil is evident in the $^{1}$H NMR spectra (Fig. 1). The data clearly indicate that conversion of triglycerides to methyl esters occurred efficiently. Similar results were also obtained for the remaining accessions (data not shown).

Five common major fatty acids of plant oils include palmitate (16:0), stearate (18:0), oleate (18:1), linoleate (18:2) and linolenate (18:3). FAC of a vegetable oil profoundly influences the quality of biodiesel; since the fuel properties such as fuel ignition quality, cold flow, heat of combustion, oxidative stability and viscosity depend mainly on the FAC of an oil, particularly high level of MUFA namely oleic acid (Knothe, 2005; Qu et al., 2012; Ullah et al., 2014). Keeping in view, the FAME samples as prepared in the study were used in FAC analyses by gas chromatography (Fig. 2), and the details are shown in Table 2. The descriptive statistical data are presented in Table 3. Four major fatty acids namely palmitic, stearic, oleic and linoleic acids were found in all the *J. curcas* accessions, and their contents ranged from 8.64 to 17.05%, 4.34 to 7.94%, 26.26 to 46.36% and 28.72 to 53.78%, respectively. The variances for the individual fatty acids are as follows: 4.0 for palmitic, 0.81 for stearic, 32.9 for oleic and 36.44 for linoleic acids. The total unsaturated fatty acid (UFA) content across the *J. curcas* accessions varied from 75.08 to 84.90% which were consistent with the Mexican accessions (59.14–86.76%), and the accessions collected in India (63.64–89.10%) as reported earlier (Ovando-Medina et al., 2011; Sinha et al., 2016). On the basis of FAC analysis, the *J. curcas* accessions could be distinctly grouped into two categories: a) oleic acid content was found to be more than linoleic acid in 9 oil samples, and the values ranged from 40.02 to 46.36%, b) high level of linoleic acid (more than 45%) was found in 6 oil samples. Various factors such as genotype, agronomic practices, edaphic and climatic conditions are considered to be the major contributing factors for variation of both seed oil content and FAC between the *J. curcas* accessions (Montes et al., 2015; Negussie et al., 2016). The impact of these factors could be understood through multi-location trials and performance evaluation of the individual accessions.

## 4. Conclusions

Biodiesel has been identified as a promising renewable fuel source in many countries. In this study, seed oil content, oil quality in terms of FFA content and FAC were analyzed for selection of the superior *J. curcas* genotypes. Most of the *J. curcas* accessions reported here could be grouped into two categories. Some of the seed oils were oleate-rich suitable for biodiesel feedstock; whereas others were linoleate-rich useful for other industrial applications. Apart from clonal propagation, field trials and large-scale plantations, these promising *J. curcas* accessions will be useful as prebreeding materials in various crop improvement programs.

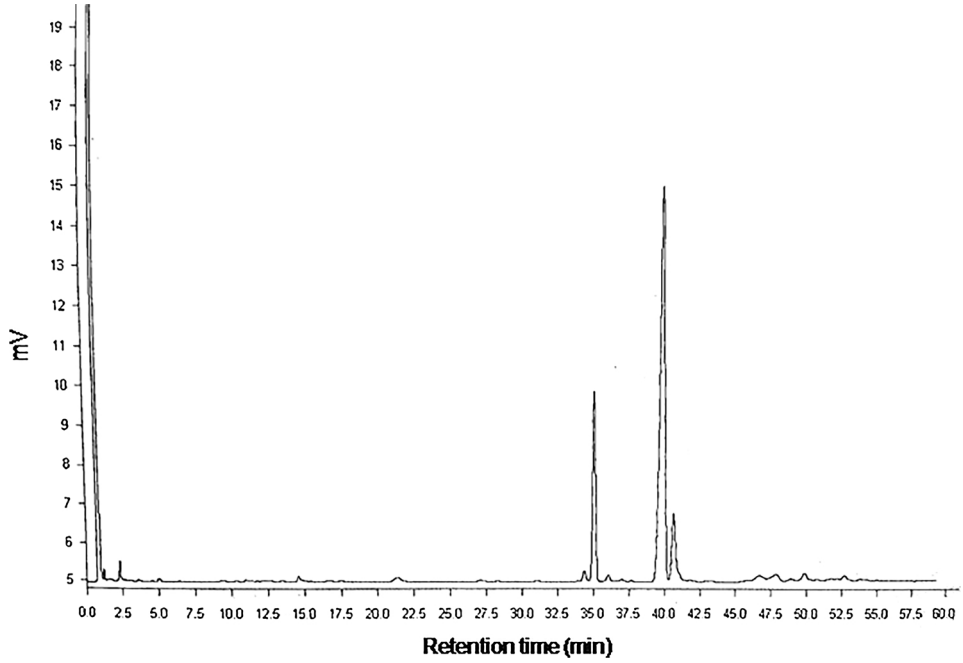


**Fig. 2.** GC chromatogram for the biodiesel sample (*J. curcas* accession TJS-01#04).

**Table 3**
Descriptive statistical analysis on the basis of fatty acid composition of the seed oil samples from the *J. curcas* accessions.

| Fatty Acid | N | Mean | Minimum | Maximum | SD | Variance | Range |
|---|---|---|---|---|---|---|---|
| Palmitic Acid | 19 | 12.38 | 8.64 | 17.05 | 2.00 | 4.00 | 8.41 |
| Stearic Acid | 19 | 5.72 | 4.34 | 7.94 | 0.89 | 0.81 | 3.60 |
| Oleic Acid | 19 | 38.45 | 26.26 | 46.36 | 5.73 | 32.90 | 20.10 |
| Linoleic acid | 19 | 41.99 | 28.72 | 53.78 | 6.03 | 36.44 | 25.06 |

## Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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