Somatic embryogenesis, molecular characterization and genetic transformability of the Kenyan Jatropha (*Jatropha curcas* L.*)*

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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DEDICATION

This work is dedicated to my late beloved father for his blessings. May God rest his soul in eternal peace. To my mother, you brought me into being. To my siblings despite missing my presence during the entire process of making this dream a reality, you believed in me and offered support when I needed it, I love you all. Above all, glory be to almighty God for favour, strength, peace, provision and good health during the entire research process.

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ABBREVIATIONS

ACMV	African Cassava Mosaic Virus
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analyses of Molecular Variance
BAP	6- benzylaminopurine
CIM	Callus inducing medium
CTAB	Cetyl trimethyl ammonium bromide
cTBP	C-terminal Binding Protein
DNA	Deoxyribonucleic Acid
dNTP	2‟, 3‟-deoxyribonucleoside 5‟-triphosphate
EDTA	Ethylenediamine tetra-acetic acid
EU	European Union
FACT	Fuel on Agricultural Common Technology
FAO	Food and Agriculture Organization of the United Nations
GAF	Green Africa Foundation
GUS	β-glucuronidase
IAA	Indole -3-acetic acid
IBA	Indole-3-butyric acid
IBR	Institute for Biotechnology Research
ISSR	Inter simple sequence repeat
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEFRI	Kenya Forestry Research Institute

KIN	Kinetin
MS	Murashige and Skoog
NAA	Naphthalene acetic acid
NGO	Non Governmental Organization
NMK	National Museums of Kenya
OD	Optical density
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RIM	Rooting inducing medium
RT	Room temperature
SIM	Shoot inducing medium
Taq	Thermophilus aquaticus
TBE	Tris borate EDTA
TDNA	Transfer DNA
TDZ	Thiadiazuron
TE	Tris EDTA
Tris	Tris (hydroxymethyl) aminomethane
UPGMA	Unweighted pair group method of arithmetic averages
VF	Vanilla Foundation

ABSTRACT

Jatropha *(Jatropha curcas* L.*)* is considered a potential biofuel crop as it contains high amounts of oil in its seeds and has potential for other applications in agriculture and health. The major factors affecting Jatropha production include poor germplasm that is prone to pests and diseases, lack of knowledge on appropriate production methods and low genetic diversity, lack of quality planting material and lack of supporting government policies. The objectives of this research were to collect, document cultural and agronomic practices, characterize, regenerate Kenyan germplasm and carry out basic genetic transformation geared towards improvement for oil production.

A total of 96 accessions were collected whereby 43.75 % were from Eastern, 16.67 % Rift Valley, 7.29 % Nairobi, 14.58 % Central, 10.42 % Western and 7.29 % from Coast. Production constraints recorded included pests and diseases, lack of market, unreliable rains, lack of quality planting material and limited management practices. *In vitro* regeneration showed that combination of 1.5 mg/l BAP, 0.6 mg/l Kinetin, 0.5 mg/l IAA and 0.1 mg/l Thiadiazuron gave best callus formation, shoot regeneration in the 4 accessions tested while 3.0 mg/l IBA and 3.5 mg/l NAA gave the best root formation.

Accessions from Rift Valley showed the widest morphological diversity while those from Eastern and Coastal showed the least diversity. The phylogenetic and PCA analyses clustered the 69 accessions into three main clusters, Central, Coast and

Western, Eastern, Rift valley and Nairobi accessions. GUS assay confirmed that the transformation efficiency was 44.43%.

Jatropha production in Kenya suffers a wide range of production challenges which have attributed to its poor performance. Somatic embryogenesis of Jatropha was successfully established.

The morphological and genetic differences among the accessions revealed by clustering into distinct groups suggest the presence of different sources of variations among the Jatropha accessions. This could be attributed to their diversity, geographical locations and also due to exchange of plant genetic resources among farmers within and between the regions. The moderate morphological diversity observed within the accessions points to ample possibilities of obtaining desirable trait combinations in Kenyan Jatropha. Available diversity and agronomic practices documentation can be used in future improvement of Kenyan Jatropha germplasm. This wide diversity can also be utilized in the selection of promising parents and inbred line development. Proper conservation of accessions studied could serve as raw material for the genetic improvement of different characters of the crop through recurrent selection after hybridisation. *In vitro* protocol regenerated developed could be used in production of elite accessions identified in mass for farmers. Transformation for specific traits should be done using the established transformation protocol to improve Jatropha production.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Description of Jatropha

Jatropha curcas L. commonly known as physic nut or purging nut is a dicotyledonous, perennial small tree usually 3 - 6 meters tall, but can attain a height of up to 8 or 10 meters under favourable conditions with effective yield for 50 years (Singh *et al*., 2007). It belongs to *Euphorbiaceae* family, genus *Jatropha,* which is thought to comprise approximately 170 known species with ploidy levels ranging from 2n to 4n (Heller, 1996). The species *Jatropha curcas* is derived from Greek words *jatrós* meaning doctor and *trophé* meaning food (Dehgan, 1979). Its genomic size is estimated to be 416 Mbp (Carvalho et al., 2008). This is relatively a small size and could make Jatropha a good candidate for sequencing (Zonneveld *et al.,* 2005).

The stems are thick, green, glabrous, mostly herbaceous or succulent, becoming woody at the base. It has spreading branches and stubby twigs, with a milky or yellowish rufescent exudate. Leaves are alternate but apically crowded, of ovate, acute to acuminate shapes and truncate to cordate at the base with 3 to 5 lobes on the outline. The leaf length and width vary from 6 – 40 cm and 6 – 35 cm respectively; the petioles are 2.5 – 7.5 cm long and of deciduous nature shedding leaves in the dry season (Henning, 2007a).

*Jatropha curcas (*Jatropha) is a monoecious with male and female flowers on the same plant and in the same inflorescence. The size of the inflorescences can vary considerably from 5 – 9.5 cm in length and 4.5 – 12.5 cm in diameter and with this variable size, the number of flowers also varies. Although the number of male to female flowers per inflorescence varies a lot among Jatropha, it was observed that male flowers were 25 – 238 and female flowers were 1–19, giving the male-to-female flower ratio of 22:1 to 29:1(Bhattacharya *et al.,* 2005). Male flowers are small and plate shaped with five sepals of 5 mm long and five petals of 7 mm. The petals convene at the flower base to form a short tube. They have ten diadelphous stamens arranged in two tiers of five with the lower tier free, while the upper tier is united. The anthers are yellow and approximately 2 mm long (Raju *et al.,* 2002; Chang-wei *et al.,* 2007). The flowers are both insect and self pollinated; insect's pollinators belong to the order of the Hymenoptera. On male flowers, bees contributed to 34 % visitors, ants 61 % and flies 5 % while on female flowers; bees make up 28 %, ants 70 % and flies 2% of the total (Raju *et al.,* 2002). Another report showed a major abundance in the *Apis* genus honey bee of 71 % pollinators (Bhattacharya *et al.,* 2005).

Flowering: occurs during the wet season and two flowering peaks are often observed although in permanently humid regions it occurs throughout the year. In good rainfall conditions nursery plants flower after the first rainy season while direct sown plants after the second rainy season. Flowers of Jatropha produce scented nectar hidden in the corolla and only accessible to insects with a long proboscis or tongue. Female flowers open one or two days before the male ones or at the same time as the earliest

male flowers. Male flowers last only for one day. Plants raised from seed are more resistant to drought than those raised from cuttings, because they develop a taproot and fibrous roots respectively. Plants from cuttings produce seeds earlier than plants grown from seed (Makkar *et al*., 2001). On average fruit production starts from the second year and stabilizes by the fourth year (Heller 1996; Openshaw 2000). In good soils it gives about 2 kg of seed per plant while in poor soils; the yields have been reported to be about 1 kg per plant (Singh *et al*., 2007).

Fruiting: It occurs several times during the year in good soil moisture. Each inflorescence yields a bunch of approximately ten or more ovoid fruits. The exocarp remains fleshy for a period of 80 – 100 days until the seeds mature. Three, bi-valved cocci are formed after the seeds mature and the fleshy exocarp dries. Seeds become mature when the capsule changes from green to yellow, then black after two to four months (Morton, 1977; Little *et al.,* 1974). The seeds are approximately 18 mm long and 10 mm wide. Jatropha has a potential production lifespan of 30 -50 years (Dehgan, 1979) with most of the seeds being poisonous although some varieties are edible especially after roasting (Makkar, 1998). The seed weight per 1000 seeds is 400 - 730 g. Although seed viability deteriorates with time, when well stored at 16°C, an average germinating capacity of about 50 % can be achieved even after 7 years. Viable seeds take 10 days to germinate. Seeds from fruits that are left on the ground surrounding the mother plant seldom germinate and develop due seed nature. The fruit and seeds (Plate 1.1) are poisonous and are not eaten or collected by animals, therefore

are not naturally dispersed. Cytogenetic studies in the genus Jatropha show that chromosome numbers are diploid with 2n = 22 chromosomes (Nair *et al.,* 2009).

Plate 1. 1: Flower head, ripe and unripe fruit of Jatropha, (Muok, 2008).

1.2 Jatropha Production and utility

Worldwide biofuel are attracting interest as substitutes for fossil fuels in order to address energy cost, energy security and global warming concerns. Fossils fuel sources are getting depleted due to large scale use. Pollution produced by their combustion has caused ecological problems like greenhouse effect leading to global climate change which is a World concern. Biofuel production from Jatropha is valued as it is a carbon credit generating tree which recycles 100% of the CO_2 emissions produced by burning the biodiesel made from it.

Jatropha a non edible oil yielding plant takes a special place in contest for biofuel crops, as it is claimed to enhance socioeconomic development through biofuel production while controlling soil erosion, reclaiming marginal and degraded lands in semi-arid regions (Francis *et al.,* 2005). It does not compete with existing food crops to produce biodiesel or deplete natural carbon stocks and ecosystem services. Global biofuel interests increased due to overestimated sustainability claim of the Jatropha biofuel (Fairless, 2007). This triggered large-scale investments and expansion of Jatropha plantations in several countries (Carels, 2009; Gexsi, 2008). Knowledge on the impacts and potentials of Jatropha plantations in developing countries is limited hence need for research (Achten *et al.,* 2007).

Jatropha is native to Mexico and Central America but it is widely distributed in wild or semi cultivated stands in Latin America, Africa, India and South East Asia (Henning, 2008; Jepsen *et al.,* 2006). In Kenya, it was introduced by Portuguese in the 16th century and occurs in many parts where it's generally grown as a fence. Jatropha is desired due to its drought hardiness, rapid growth, easy propagation, low seed costs, high oil content, small gestation period, wide adaptation, production on good and degraded soils and the plant height that makes the seed collection more convenient (Francis, 2005; Jones, 1991).

Jatropha which is a multi-purpose plant has historical records showing that it was used by native Indians of Central America and perhaps South America as herbal medicine. Its seeds are rich in oil and when extracted, its pure oil can be used directly to produce

light, warmth and electricity or it can be used as a feedstock for bio-diesel. For this reason, Jatropha has rapidly been introduced in commercial plantations and various rural development programs, as it may contribute to income generation and increase efficiency of rural and agricultural processes.

Jatropha enclosure of fields can contribute to a reduction of conflicts between herders and farmers. Herders often tie their goats to Jatropha bushes in Cape Verde instead of free range grazing where they can be destructive to farmers (Münch and Kiefer, 1989). Jatropha can flower three times a year where bees pollinate them thus it is possible to have apiaries in association with Jatropha. The use of Jatropha seeds, leaves and oil as a folklore medicine for rheumatic pains and snake bites has also been documented (Neuwinger, 2000). The latex of Jatropha has a widespread reputation for healing wounds, as a haemostatic, curing skin problems and having anti-cancerous properties (Kosasi *et al.,* 1989). Leaf juice is used to cure scabies, eczema and ringworms (Rajore *et al.,* 2005). The bark of Jatropha yields a dark blue dye which is used for colouring cloth, fishing nets and lines. The leaves are used as food for the tusser silkworm. It can also be used to alleviate soil degradation, deforestation and desertification. According to Jongschaap, (2007), Jatropha can also reclaim marginal soils, conserve, protect and improve soils, protect against erosion by use of its dense, wide-ranging root structure. It is also useful in the production of CO_2 neutral biofuels.

1.3 Economic Importance

In 1836 Jatropha seeds were commercially produced in Cape Verde Islands and exported to Portugal and France for oil production which was used for street lighting and soap production (Francis, 2005; Jones, 1991). Nowadays the oil is either used directly in adapted engines powering local grain mills, water pumps and small generators or first refined by transesterification with methanol or ethanol to produce regular fuel suitable for high performance diesel engines (Fangrui, 1999). The oil is also used as an illuminant as it burns without emitting smoke. Its importance also lies in its medicinal properties because of its insecticide and molluscide effect, the oil can be used as a natural crop pesticide. In some countries such as Tanzania and Mali, the oil is extensively used for making soap due to its high saponification value. Traditionally, soap has been produced from Jatropha oil in several countries. High-quality soap can only be produced with modern production methods. The oil cake is rich in nitrogen, phosphorous and potassium and can be used as organic manure. Other levels of use can be exploited like direct fermentation of seed cake and pulp to deliver an organic fertilizer that has a high potential for export to developed countries. An example of an economic potential project is Kakute Project in Tanzania, Arusha consisting of Maasai women and a women group in Mtu Wa Mbu who make and sale the "medicinal soap" to the rural population (Henning, 2007b).

1.4 Jatropha production constraints

When Jatropha grows as a solitary plant or in small stands it rarely shows signs of pests and diseases. However, when cultivated in higher densities in plantations or hedges this situation changes with the number of infections increasing. When a new crop is introduced and cultivated on a large scale, it can take years before the pest and disease pressure is felt. The low incidence rate of pests and diseases currently observed in most areas cannot be assumed to be a degree of pest and disease resistance (Achten *et al.,* 2008).

Important pests and diseases that have been reported to affect Jatropha vary with regions. In Africa: Flea beetle (*Aphthona spp.)* eats the leaves and their larvae penetrate the roots (Nielsen, 2007). The yellow flea beetle (*Aphthona dilutipes*) appears to cause more severe damage than the golden flea beetle, sometimes resulting in 100% mortality of leaves and seeds. Yellow flea beetle has been observed in Mozambique and Malawi where it causes severe damage. In Central and South America: fruit feeding true bugs, *Pachycoris klugii Burmeister* (Scutelleridae) and *Leptoglossus zonatus* (Coreidae) have been reported (Grimm and Maes, 1997).

In Asia the scutellarid bug (*Scutellera nobilis*) which causes flower fall, fruit abortion and malformation of seeds, and the inflorescence and capsule borer (*Pempelia morosalis)* that causes damage by webbing and feeding on inflorescences and in later stages boring in capsule have been reported by Shanker *et al.,* 2006. In India virus damage is of major concern and appears to be spreading fast while in Africa virus

presence is rare. There is concern that, for instance, African Cassava Mosaic Virus (ACMV) may be transferred by Jatropha (Münch *et al.,* 1989) due to the fact that ACMV is transmitted by whitefly (*Bemisia tabaci*) also found in Jatropha plants. For these reasons it is advised not to plant cassava and Jatropha in the same field (Heller, 1996). Common bean (*Phaseolus vulgaris)* is also susceptible to Jatropha Mosaic Virus (Hughes *et al.,* 2003). Currently there is no knowledge about the efficiency of pests and disease control methods hence there are no recommended approaches (Grimm1999, Raj *et al.,* 2008). However, control methods that work with other crops may be efficient in Jatropha too. For example some pesticides have been used successfully against major pests in Jatropha which include Chlorpyrifos or Cyphenothrin against *Aphthona spp*. (Nielsen, 2007) and Captafol in eradication of ACMV (Lozano *et al.,* 1981),

Preventive measures are by use of resistant Jatropha varieties where presently there is no systematic knowledge about resistant varieties; however, healthy plants should be selected as "mother plants" for seeds and cuttings. This should be accompanied by avoiding planting Jatropha when the pest pressure is high normally towards the end of the rainy season when temperatures and relative humidity are high (Bhattacharya *et al.,* 2005). Disinfecting tools used for cutting and pruning with alcohol and chlorine is quite efficient. Weekly inspection and uprooting diseased plants, drying, burning or burying them far to prevent whiteflies is recommended. Minimizing damage to the plants to reduce the risk of microorganisms entering by pruning with sharp tools only and always cut at an angle to avoid creating horizontal cuts where water will drain

slowly. Use of wider spacing of 3m by 3m within and 4 m between rows and intercropping to reduce incidence of pests is recommended (Nielsen, 2007).

1.5 Statement of the problem and justification

Production of *Jatropha curcas* in Kenya is important due to its potential as biofuel crop. Although there are enormous claims about Jatropha such as low cost of production, short maturity period, ability to grow in both good and degraded soils as well as in low and high rainfall areas, and that it is resistant to pests and disease; theses resistances have not been proven. Current knowledge gaps and uncertain economic perspectives might drive Jatropha investors away from farming in marginal or degraded lands to agricultural or lands that are valuable for biodiversity, in order to reduce financial risk.

Jatropha production in Kenya encounters serious problems including lack management practices, pest and diseases, lack of quality planting material, lack of information on the genetic base and low genetic diversity. Some pests and diseases have been observed on Jatropha in Kenya which include powdery mildew, which damages leaves and flowers, Alternaria, which causes premature leaf fall and golden flea beetles, which eat young leaves and shoots. It is also a host for cassava viruses which cause total loss of young seedlings. These problems have limited commercial production of Jatropha in most growing regions of the country.

Although Jatropha was introduced into Kenya in the 16th century by the Portuguese navigators there are no records of the species origin, where it was first planted and the

additional introduction made due to poor record keeping (Muok and Kallback, 2008). Little documentation exists on the collection, distribution, characterization and improvement of the Kenyan Jatropha germplasm. Additionally, farmers lack quality planting materials. Therefore, there is need to document, characterize and start improvement programs of this crop. Moreover the seed oil content is not predictable varying from 4 to 40% due to lack of improved varieties. There are no established Jatropha morphological descriptors by international board for plant genetic resources (IBPGR) so far making morphological characterization a challenge.

Jatropha has assumed paramount importance as a potential biodiesel crop in more than 50 countries worldwide. Despite the many claims it has Jatropha just like any other crop, needs resources to achieve high productivity. If Jatropha competes for land with food crops or high carbon stocks it would lose its acclaimed sustainability advantages. The considerable lack of insight in genetics, input responsiveness and agronomy makes yields poorly predictable (Achten *et al.,* 2008). Additionally, monocultures are likely to face unexpected pest and disease infestations (Shanker and Dhyani, 2006).

Jatropha production for biofuels in Kenya is very attractive due to its hardiness and stress handling ability. This would lead to economic empowerment, social upliftment and poverty alleviation within marginalized communities.

To meet large scale demand and ensure continuous supply of elite planting material there is need to establish clonal seed orchards and develop mass production techniques through tissue culture. Protocols for high frequency *In vitro* regeneration of Jatropha

have been developed for Asian genotypes but none has been reported for Kenyan genotypes. All these *In vitro* regeneration protocols have also not been translated into efficient genetic transformation system. Transgenic approach has the potential to significantly improve seed productivity (Gressel, 2008; Sujatha *et al.,* 2008), pests and diseases which are the current threats in Jatropha production.

Although genetic diversity in Jatropha using isozyme, random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and inter simple sequence repeat (ISSR) markers have been used to determine genetic diversity in several countries (Sujatha and Prabakaran, 2003; Basha and Sujatha, 2007; Ganesh *et al.,* 2008; Mohamud *et al.,* 2008; Subramanyam *et al.,* 2009) limited research has been done in Kenya. Research on Kenyan germplasm diversity is essential for better understanding of genetic relationships leading effective organization and management of Jatropha germplasm.

There is need therefore to determine the cultural and agronomic practices of Jatropha, distribution of the available diversity in Kenya, develop an *In vitro* regeneration protocol and test its transformability. These desertion document the work done on collection of Jatropha germplasm, of cultural and agronomic practices, characterization, tissue culture and genetic engineering.

1.7 Objectives

1.7.1 General objective

To enhance Jatropha production in Kenya.

1.7.2 Specific objectives

1. Documentation of agronomic and cultural practices of Jatropha in Kenya.

2. Development of a regeneration protocol for Jatropha micropropagation.

3. Morphological characterization of Jatropha germplasm found in Kenya.

4. Molecular characterization of Jatropha germplasm found in Kenya.

5. Evaluation of Jatropha transformability using *Agrobacterium tumefaciens.*

1.8 Hypotheses

1. There are no documented agronomic and cultural practices on Jatropha farming in Kenya.

2. There is no genetic diversity in Kenyan Jatropha germplasm.

3. Jatropha *In vitro* regeneration and *Agrobacterium tumefaciens*-mediated transformation are not possible.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin, distribution and ecology of Jatropha

Jatropha curcas is possibly a native of Mesoamerica region that is Mexico and Central America (Dehgan *et al.,* 1979; Openshaw, 2000). Nowadays this species is distributed throughout the tropical and subtropical world, as a result of the European colonialism the plant was introduced to Caribbean islands, Africa and Southeast of Asia where it is grown as a hedge plant. Nevertheless, there are disagreements and some authors consider Brazil as its origin, as suggested by Oliveira *et al.,* 2006. Its extensive culture in Brazil has led to the recommendation that it's a native to the South American country (Melo *et al.,* 2006).The State of Ceara, in Brazil, is mentioned to be the centre of origin of the plant . In the same way, Basha *et al.,* 2009; Sudheer *et al.,* (2009b) and (2010b) mention that this plant is a native of South America. Basha *et al.,* 2007, declare that it is a native of Mexico and the Central American region. Other authors prefer amore conservative point of view stating that the origin of the plant is "tropical America" (Ambrosi *et al.*, 2010; Divakara *et al.,* 2010; Ganesh-Ram *et al.,* 2008 and Ranade *et al.,* 2008).

Jatropha curcas is the most primitive form within the *Jatropha* genus (Dehgan *et al.,* 1979). There are a few recognized varieties of *Jatropha curcas* in the world and their differentiation is based upon the size or the content of toxic molecules of the seed. However this classification has an element of arbitrariness. For example, three

varieties are frequently mentioned by researches: the Cape Verde variety that has spread all over the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety that only has traces of phorbol esters in the fruit making it suitable for human consumption after roasting (Heller,1996; Sujatha *et al.,* 2005). Recently, some commercial varieties have been released, for example the SDAUJ1, from an Indian program for germplasm selection and JMAX, derived from Guatemalan germplasm (Basha *et al.,* 2007).

Africa has about 70 native species with Madagascar having one which is endemic, several Jatropha species are widely grown in the tropics as medicinal or ornamental plants. Some consider Jatropha to be a weed, however, it is definitely not an invasive species since it hardly germinates on its own (Dehgan *et al.,* 1979).

Today, Jatropha is cultivated and naturalized in almost all tropical and subtropical countries as protection hedges around homesteads, gardens and fields because it is not browsed by animals as a result of its toxins (Reinhard *et al.,* 2007). It is well adapted to arid, semi- arid tropical and warm subtropical climates with mean daily temperatures of 20° – 30°C and annual rainfall of 300 - 600 mm (Figure 2.1). Jatropha is susceptible to high frost and waterlogged terrain, but tolerant to periods of droughts of up to 7 months. Jatropha can grow on degraded, sandy and even saline soils with low nutrient content. In Kenya Jatropha is mainly grown in Eastern, Central, Rift valley, Coastal and Western regions (Muok *et al.,* 2008).

However, for economically sustainable oil production, well drained soils of good physical and chemical quality with at least 750 mm annual rainfall or supplementary irrigation is recommended (Henning, 2007a).

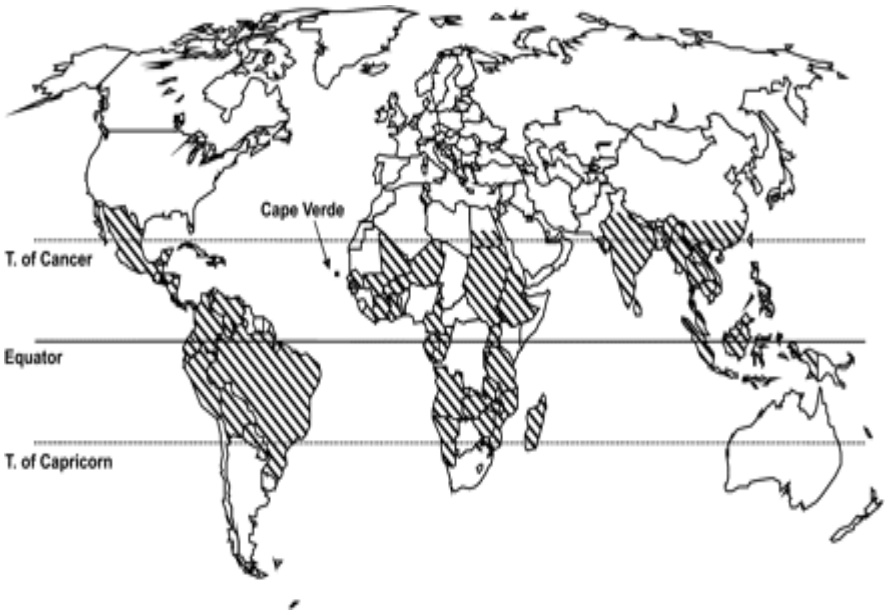


Figure 2.1: Global distribution of Jatropha, (Heller, 1996).

2.2 Agronomic practices

Jatropha can be established from seeds and cuttings. Plants from seeds develop a taproot and four lateral roots, whereas it has been reported that plants from cuttings do not develop a taproot (Heller *et al.,* 1996). The best time for planting is in the warm season before or at the onset of the rains. In the former case, watering of the plants is required. The recommended spacing for hedgerows is 15 to 25 cm apart. For maximum yield in plantations , spacing of 2-3 m between and 2-2.5 m within rows is applied giving plant densities of 1350-2500 plants /ha (Jones *et al.,* 1992).

It is essential that extracted seeds are partially dried under shade to improve germination since wet seeds are prone to fungal attack resulting in poor germination. Seed pre-treatment for propagation is not necessary. At nursery level, seeds do not need fertilizers to initiate root and shoot development as they have enough food reserves. Seeds just germinate when embedded in a media allowing seed interaction with light, water, warmth and air. Wet coarse sand is recommended for use in a nursery bed. After germination a nutrient rich media is required with good level of organic matter. This media should be used in appropriate potting tube size dependent on expected seedling duration in a nursery bed, customer needs and plant size. Customized watering regime should be administered to potting bags with sown seeds thereafter. When seedlings obtained either from sown seeds or cuttings are rooted in nurseries for 4-6 months, higher survival rate is recorded than those directly propagated to fields. Protection of young seedlings against ruminants is required as the leaves and shoots will have not yet developed repellent toxins (Achten *et al.,* 2008).

Jatropha usually survives when overgrown by weeds, but growth and production is minimal. Weeding improves soil fertility, prevents nutrient and light competition. It can stop after dense canopy formation which severely suppresses weeds hence labour for weeding consequently drops (Flemming, 2007).

Flower and seed production respond well to rainfall and availability of nutrients. Jatropha needs sufficient amounts of nutrients in order to grow into a full size plant and produce seeds. In the first 4 years nutrients are needed to build up a good plant

architecture and production of flowers and fruits. Afterward nutrients are mainly needed for maintenance of the plant and for fruit production. These can be from its fruit shell and press cake or nitrogen, phosphorus and potassium fertilizers. In case of highly fertile soils, this is not necessary (Gubizt *et al.,* 1997; Janssen, 1991).

Jatropha flowers form only at the end of branches, pruning leads to more branches, fruit production and easy to harvest by hand due to low height. Good pruning avoids competition for light and space forming strong lateral branches that can bear the weight of the fruits. Pruning should be done under dry conditions to reduce the risks for bacterial or viral plant infection and fungal attacks (Medina *et al.,* (2011).

As Jatropha plants are often neglected in the first year(s) due to low return, intercropping is recommended if nutrients and water are available. Intercropping nitrogen fixing species, with annual or biannual crops that remain relatively low like corn, peanuts, beans and peppers is recommended. Jatropha should not be intercropped with cassava, since it is a possible host for several cassava diseases (Raj *et al.,* 2002).

2.3 Germplasm characterization

Determination of genetic relationships among species is critical for the management of genetic resources and success of interspecific hybridization. Molecular markers reveal more quickly and accurately, genetic differences far exceeding those obtainable using morphological or biochemical methods without the obscurance of environment. Molecular markers are not typically influenced by environmental conditions and

therefore can be used to help describe patterns of genetic variation among plant populations and to identify duplicated accessions within germplasm collections. Molecular markers are identifiable DNA sequences, found at specific locations of the genome, transmitted by standard Mendelian laws of inheritance from one generation to the next. They rely on a DNA assay and a range of different kinds of molecular marker systems exist, such as amplified fragment length polymorphism (AFLP) (Zabeau *et al.,* 1993), restriction fragment length polymorphism (RFLP) (Botstein *et al.,* 1980), simple sequence repeats (SSRs) (Tautz ,1989) and randomly amplified polymorphic DNAs (RAPD) (Williams *et al.,* 1990).

This technology has improved in the past decade and, fast, cheaper systems like single nucleotide polymorphisms (SNPs) are increasingly being used. The different marker systems may vary in aspects such as their technical requirements, the amount of time, money and labour needed and the number of genetic markers that can be detected throughout the genome. Molecular markers have been used in laboratories since the late 1970s and they are applied across the food and agricultural sectors. They are very versatile and can be used for a variety of purposes. Thus, they are used in genetic improvement, through the so-called marker-assisted selection (MAS), where markers physically located beside or even within genes of interest are used to select favourable variants of the genes (Guimarães *et al.,* 2007). Molecular markers are also used to characterize and conserve genetic resources, where some of the approaches can be applied in each of the crop, forestry, livestock and fishery sectors like estimating the genetic relationships between populations within a species.

Molecular markers can be used to identify duplicate accessions in crop gene banks; monitor effective population sizes or carry out biological studies like mating systems, pollen movement and seed dispersal in forest tree populations (Ruane *et al.,* 2006a). They are also used in disease diagnosis, to characterize and detect pathogens in livestock, crops, forest trees, fish and food. Molecular markers have been used in a number of developing countries. In crops, new hybrids and varieties have been developed using Marker Assisted Selection such as pearl millet, rice and maize (Varshney *et al.,* 2006). The use of RAPD markers is cheap and a rapid method not requiring any information regarding the genome of the plant, and has been widely used to ascertain the genetic diversity in several plants (Belaj *et al.,* 2001; Deshwall *et al.,* 2005). RAPD analysis requires only a small amount of genomic DNA and can produce high levels of polymorphism and may facilitate more effective diversity analysis in plants (Williams *et al.,* 1990). RAPD analysis provides information that can help define the distinctiveness of species and phylogenetic relationships at molecular level. Use of such techniques for germplasm characterization may facilitate the conservation and utilization of plant genetic resources, permitting the identification of unique genotypes or sources of genetically diverse genotypes. RAPD analysis has been used for genetic diversity assessment and for identifying germplasm in a number of plant species (Kapteyn and Simon, 2002; Welsh and McClelland, 1990).

Jatropha is a diploid species with a genome size of 416 Mbp which is relatively small for a plant genome and could make it an attractive candidate for genome sequencing (Carvalho *et al.*, 2008; Zonneveld *et al.*, 2005). Attempts have been made to assess the

extent of genetic variability in Jatropha germplasm using morphological and molecular markers for seed and oil content traits which show significant variability for these traits (Kaushik, 2007). Molecular characterizations have been carried out using RAPD, ISSR and AFLP markers. These studies were confined to accessions available in India whereby variation reported was mainly due to inclusion of wild species or geographical background (Basha, 2007; Ganesh, 2008; Ranade, 2007). In India low degree of variation among the accessions collected from different geographical regions has been reported using both RAPD and ISSR markers (Basha *et al.,* 2007).

Genetic background of 72 Jatropha accessions representing 13 countries was elucidated using molecular and biochemical analyses. The biochemical composition analysis of seeds showed wide variation in crude protein, oil and ash content and phorbol ester in seed. However, simple sequence repeat (SSR) analysis found out that accessions from many countries failed to be distinguished with the exception of accessions from Mexico and El Salvador (Basha *et al.,* 2009). A recent study used RAPD, AFLP and C-terminal Binding Protein (cTBP) to assess genetic polymorphism in Jatropha accessions from 13 countries on 3 continents and found a high degree of monomorphism in Jatropha accessions while Mexico and Costa Rica accessions exhibited polymorphism. Overall, clear phenotypic variation among Jatropha collections is well established. However, high phenotypic variation is matched by low level of genetic diversity as revealed by morphological markers (Popluechai *et al.,* 2009).

2.4 *In vitro* regeneration of Jatropha

In the recent years *In vitro* culture techniques have attracted a lot of interest as they offer a viable tool for mass multiplication and germplasm preservation of rare, endemic and endangered plants (Villalobos and Engelmann, 1995). These technologies could offer a cost effective production of elite planting material in high volumes throughout the year without seasonal limitations (Cassell *et al.,* 1999).

In vitro culture is the technique of growing plant cells, tissues and organs in an artificial prepared solid or liquid nutrient medium, under aseptic conditions and controlled environment. These ranges from protoplasts, single cell to unorganized mass of cells (callus) or highly organized multicellular cells (tissues). Plant regeneration through tissue culture can be accomplished using one of the three methods: meristem culture, somatic embryogenesis and organogenesis (Thorpe *et al.,* 1990). Micropropagation of tree species offers a fast means of producing clonal planting stocks of elite, rare and endangered genotypes hence boosting their production. It is a useful tool for selection, development of new cultivar, conservation of germplasm and genetic manipulation. This technique has been applied to tree species that are difficult to establish conventionally like those with recalcitrant seeds and where demand for seedlings is high (Karkonen *et al.,* 1999).

Micropropagation: This process consists of several stages namely preparation and pretreatment of the plant, initiation of the explants, multiplication of the tissue, regeneration of whole plants and hardening for subsequent field planting (Geroge *et*

al., 1984; McCown *et al.,* 1987). The choice and pretreatment of the tissue to be propagated is of crucial importance and determines the success or failure of subsequent initiation phase. Plants from a controlled environment are preferred to open fields as they have low levels of fungal/bacterial/viral contaminations. Initiation of explants is more successful from healthy and vigorous growing material. Some species like bulbs and trees require treatment like high temperatures to break dormancy (Van *et al.,* 1986). Initiation of explants is mostly achieved on Murashige and Skoog (MS) , with a number of variations although in some plant genera growth hormones will be required. Tissue multiplication requires cytokinins alone or in combination of auxins at low levels. Regeneration of whole plants entails axillary shoot proliferation and root production. This requires use of high auxins and low cytokinins levels. Micropagation has several advantages including rapid multiplication, selection and introduction of new varieties, disease indexing and eradication, improvement of plant morphology like colour of ornamental plants through mutation (Michael, 1992).

Pathway taken by the cultures is dependent on the explant type and plant growth regulator used at a given time of growth (Salvi *et al.,* 2001). Meristem and somatic embryogenesis are some of the main ways of *In vitro* plant regeneration.

Somatic embryogenesis: is an indirect way involving initiation and induction of callus formation on a non meristematic part of the explants. Callus is actively dividing non-organized masses of undifferentiated and differentiated cells often developed in

the presence of growth regulators. Callus culture technique is usually on solidified medium and initiated by inoculation of small explants. Callus cultures can be indefinitely maintained through regular sub-culturing (Merkle, 1998). Calli differ in texture and colour. The colour can vary from yellow, white and green to brown while the texture can be compact to friable and smooth to very nodular. Formation of shoots from callus is induced by lowering the auxin levels (Becwar *et al.,* 1988). Sometimes formation of direct shoots and callus may occur on the same explants simultaneously. Somatic embryogenesis normally takes place in two stages; induction of cells with embryogenic competence in presence of high levels of auxins and the development of embryogenic masses into embryos in the absence or presence of low auxin concentrations (Nadar *et al.,* 1978).

Clonal propagation by tissue culture from hypocotyls, petiole or leaf explants is important for production of genetically superior planting material. A number of plant regeneration protocols of Jatropha have been documented. Regeneration from hypocotyl, petiole and leaf using 6- benzylaminopurine (BAP) and indole-3-butyric acid (IBA) (Sujatha *et al.,* 1996) and later improved by additional use of kinetin (KIN) and thidiazuron (TDZ) on axillary buds (Sujatha *et al.,* 2005). Addition of indole -3-acetic acid (IAA), adenine sulphate, glutamine and IBA to BA on regeneration through shoot tips has also been reported (Shilpa *et al.,* 2005). Regeneration through somatic embryogenesis has also been done using KIN and IBA (Timir *et al.,* 2007) and BAP, naphthalene acetic acid (NAA) and IBA (Shilpa *et al.,* 2007). Organogenesis using nodal explants in MS supplemented with BAP, adenine Sulphate,

KIN, IBA (Datta *et al.,* 2007) and BAP, KIN and IAA (Kalimuthu *et al.,* 2007) has been achieved. High frequency of regeneration through callus using TDZ, BAP, IBA, KIN, IAA and gibberellic acid (GA_3) (Deore *et al.,* 2008) and through nodal explants using BAP, IBA, adenine sulfate , glutamine, arginine, citric acid and NAA (Shrivastava *et al.,* 2008). Shoot regeneration of Jatropha has been successfully obtained from cotyledon, petiole, hypocotyl, epicotyl, and leaf tissue (Sujatha and Mukta 1996; Lin *et al.,* 2002; Lu *et al.,* 2003; Wei *et al.,* 2004; Rajore, 2002). Regeneration has widely been reported as one of the prerequisite for genetic transformation (Birch, 1997). Further reports indicating the role of regeneration as a pillar to transformation have also been documented (Hoekema *et al.*, 1983).

2.5 Transformation of Jatropha

Plant transformation is the introduction of a foreign piece of DNA, conferring a specific trait, into host plant tissue. The foreign gene termed as the "transgene" is incorporated into the host plant genome and stably inherited through future generations. The correct regulatory sequences are added to the gene of interest (promoters and terminators) and then the DNA is transferred to the plant cell culture using an appropriate vector. The gene is attached to a selectable marker which allows selection for the presence of the transgene. Genes conferring resistance to a specific antibiotic are often used to serve this purpose. Once the plant tissue has been transformed, the cells containing the transgene are selected and regenerated back into whole plants. This is possible as plant cells are totipotent, which means they have the

capacity to form an entire plant. Therefore, the gene is contained in every single plant cell, its expression is determined by the promoter which is controlling it.

Plant transformation can be carried out in a number of different ways depending on the species of plant in question. Some important plant genetic transformation techniques are; *Agrobacterium* mediated gene transfer, biolistics, electroporation, micro injection and poly-ethylene glycol (PEG) mediated gene transfer. *Agrobacterium* mediated gene transfer involves transfer of DNA from bacteria to plants. Biolistics uses gold micro projectiles coated with DNA which are blasted into the cells. In electroporation, electrical impulses are used to increase membrane and cell wall permeability to DNA contained in the surrounding solution. Microinjection is injection of DNA directly into the cell nucleus using an ultrafine needle. PEG mediated gene transfer involves plant cell protoplasts treated with PEG which is momentarily permeable, allowing uptake of DNA from the surrounding solution (Kumar *et al.,* 2008).

Plant transformation was developed as an alternative to conventional breeding methods which are more laborious than this fairly simple laboratory procedure. Plants are transformed in order to create plants that contain genes conferring traits that are desirable to the plant breeder. This includes resistance to specific plant diseases and pests, drought and saline tolerance. It also includes novel uses of plants, such as the production of edible vaccines in plant tissue. Plants can be engineered to express sub-unit vaccines which trigger oral immunity if plant tissues are consumed as food.

Vaccines can be created to treat huge number of pathogens, such as bacteria that cause diarrhoea which causes around 3 million infant deaths per year in the developing world (Arntzen, 1998).

Plant transformation mediated by *Agrobacterium tumefaciens (A. tumefaciens)*, a soil plant pathogenic bacterium, has become the most used and cheap method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants (Smith, 1907) *A. tumefaciens* has the exceptional ability to transfer a particular DNA segment (T-DNA) of the tumor-inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into the host genome and transcribed, causing the crown gall disease (Nester, 1984). The transformation process involves a number of steps; isolation of the genes of interest from the source organism and development of a functional transgenic construct including the gene of interest; promoters to drive expression and marker genes to facilitate tracking of the introduced genes in the host plant. The transgene is then inserted into the Ti-plasmid followed by introduction of the T-DNA-containing-plasmid into *Agrobacterium.* Plant compounds like acetosyringone, ferulic acid and gallic acid are then used to initiate *Agrobacterium* to infect plant cells whereby the T-DNA is then integrated into the plant chromosomal DNA. Regeneration of transgenic plants and testing for trait performance or transgene expression at lab, greenhouse and field level then follows (Jones *et al.,* 2005).

The *Agrobacterium*-mediated transformation protocols differ from one plant species to another and within species, from one cultivar to another, consequently, the

optimization of *Agrobacterium*-mediated transformation methodologies requires the consideration of several factors that can be determined in the successful transformation of one species. First, the optimization of *Agrobacterium* plant interaction on competent cells from different regenerable tissues and then development of a suitable method for regeneration from transformed cells (Susan, 2005).

A number of studies on *Agrobacterium*-mediated transformation of Jatropha have been carried out. Establishment of the transformation via *Agrobacterium tumefaciens* on cotyledon disc explants has been reported. LBA4404 bacteria strain and phosphinothricin for selection was an improvement over the strain of EHA105 and hygromycin. About 55% of the cotyledon explants produced phosphinothricin-resistant calluses on Murashige and Skoog (MS) medium supplemented with 1.5 mg/l benzyladenine (BA), 0.05 mg/l 3–indolebutyric acid (IBA), 1 mg/l phosphinothricin and 500 mg/l cefotaxime after 4 weeks. Shoots were regenerated following transfer of the resistant calli to shoot induction medium containing 1.5 mg/l BA, 0.05 mg/l IBA, 0.5 mg/l gibberellic acid (GA_3), 1 mg/l phosphinothricin and 250 mg/l cefotaxime, and about 33% of the resistant calli differentiated into shoots. Finally, the resistant shoots were rooted on 1/2 MS media supplemented with 0.3 mg/l IBA at a rate of 78%. The transgenic nature of the transformants was demonstrated by the detection of b-glucuronidase activity in the primary transformants and by PCR and Southern hybridization analysis. Of the inoculated explants 13% produced transgenic plants after approximately 4 months (Li *et al.,* 2007). In another study LBA4404 strain

containing a neomycin phosphotransferase gene (*NPT II*), conferring kanamycin resistance and a β-glucuronidase (*GUS*) reporter gene was used. Cotyledon explants were used since they were found more susceptible to *Agrobacterium* infection than other explants such as hypocotyls, petioles or leaves (Li *et al.,* 2006).

Using MS medium with 3 mg/l BA and 0.01 mg/l IBA based on that of. According to Sujatha *et al.,* (1996), cotyledon explants of Jatropha were very sensitive to kanamycin, whereby use of 5mg/l kanamycin was sufficient to stop callus induction. Therefore, kanamycin was not included in the callus-inducing medium within the first 4 weeks during co-cultivation with *Agrobacterium* to induce calli. This was also reported by Pan *et al.,* 2010 whereby he used 20 mg/l kanamycin to select the transformants after 4 weeks of calli induction. Kanamycin-resistant regenerated shoots were obtained after 6 - 8 weeks of culture on the selective media.

The reporter gene expression was examined by histochemical staining of *GUS* activity in leaves of kanamycin-resistant regenerated shoots. To confirm the presence of the transgenes in the putatively transformed plants, genomic DNA was extracted from *GUS*-staining positive regenerated plants and a non-transformed plant for PCR amplification and Southern blot analysis. The reporter gene *GUS* was detected in all *GUS*-staining positive plants and positive control, but no amplification product was found in non-transformed control plant (Jingli *et al.,* 2010).

CHAPTER THREE

3.0 GERMPLASM COLLECTION, AGRONOMIC AND CULTURAL PRACTICES OF *JATROPHA CURCAS* IN KENYA

3.1 Abstract

In the last decade Jatropha has gained a lot of interest globally as a result of awareness of its potential as a biofuel plant. However in Kenya it is still considered a semi-wild plant and systematic crop improvement programme needs to be undertaken to exploit its utilization. Research on its germplasm collection, distribution and management are a prerequisite. The aim of this study was to document Jatropha distribution, agronomic and cultural practices and factors impeding its production.

A field survey was conducted in Coast, Rift Valley, Eastern, Nairobi, Central and Western to collect Jatropha germplasm and document its agronomic and cultural practices. Structured questionnaire were given to 100 Jatropha growers, 50 farmers interviews were carried out and personal observations noted. Germplasm in the form of seeds, stem cuttings and seedlings was also collected in the surveyed regions and documented.

Of the 96 collections 43.75% were from Eastern, 16.67% were from Rift Valley, 7.29% were from Nairobi, 14.58% were from Central, 10.42% were from Western and 7.29% were from Coast. Accessions were either land race or wild type. Use of 10 local names on areas surveyed was documented. Data on cultural and agronomic practices including land tilling, hole preparation, contour ploughing, weeding, irrigation, pests

and disease management, pruning and intercropping and production constraints were noted for each respondent interviewed. Results indicated that all farmers propagated Jatropha through seeds while 64.2% of them used both seeds and cuttings. No records of tissue culture plantlets were reported. Constraints in Jatropha production were recorded as pests like mites, beetles and whitefly attacks; diseases like powdery mildew, rust and root rot infections; lack of planting material; poor management practices and lack of market.

3.2 Introduction

Jatropha is emerging as the driver of biofuel revolution in Kenya that could also help restore the country's degenerating environment. It's said to have been introduced by the Portuguese in the 16th century and mainly used as a fence by several communities. Its cultivation started in 2005 and has been spreading in arid areas to deliver a new source of livelihood for poor communities through biodiesel production. In other parts of the world like China and India it has been used to rehabilitate dry lands. It grows well in the entire East Africa region, in Kenya, its mainly found in Western a, Rift Valley, Coast, Central and Eastern provinces. Its favoured niche in these regions is bushland and along rivers hence a reliable species for the wild, reducing competition for space with food-crops (Maundu *et al.,* 2005). Currently Jatropha is mainly grown in Kitui, Makueni, Thika, Namanga, Kajiado, Malindi, Nyanza, Nakuru, Marakwet, Marsabit, Naivasha, in the coastal regions and in Meru with the help of NGO's like Green Africa Foundation (GAF) and Vanilla Jatropha Development Foundation (VJF).

Institutions like Kenya Forest Research Institute (KEFRI) buy the harvests for conservation purposes (Isaac, 2008).

Jatropha production requires land, plantation establishment and plantation management practices (Achten *et al.*, 2008). It can grow in a wide range of soils but it is clear that it responds well on aerated soils like sandy and loam compared to clay soils. An optimal pH of between 6 and 8.5 is recommended. The plant can adapt well to marginal soils with low nutrient content however for high biomass production, the crop requires high nitrogen and phosphorus fertilization (Daey *et al.*, 2007). Jatropha cultivation improves soil resistance to wind erosion and enhanced stability to water erosion (Ogunwole *et al.*, 2008).

Propagation is mainly through seeds and cuttings. Plants raised from seed are more resistant to drought than those raised from cuttings; because of the taproot they develop (Achten *et al.*, 2007). The taproot is straight and has a deeper root system growth which extracts moisture from deeper levels of the soil. This root structure is also ideal in intercropping systems as it minimizes competition for water and nutrients between the different crops. Nursery raised seedlings have a higher survival rate compared to direct sown seedlings and produces seeds earlier. Seed treatment prior to planting is recommended (Daey *et al.*, 2007). Seed soaking in cold water for 24 hours is suggested for better and quick germination (Kaushik *et al.*, 2007). Jatropha plant densities range from 1100 to 2500 plants per hectare and wider spacing is recommended for better yields. In semi-arid regions with low-input systems wider

spacing of 3.0 x 2.0, 3.0 x 2.5 or 3.0 x 3.0 meters is applied. Spacing between rows of 2.5 meters is required to facilitate easier passage for fruit harvesting among other management practices. Planting holes of 30–45 cm wide and deep should be prepared and organic matter incorporated before planting. An insecticide may be included as a precaution against termites. The seedlings may require irrigation for the first two to three months after planting. Where Jatropha is being planted as a living hedge, cuttings of 60 – 120 cm length should be inserted between 5 and 25 cm apart and 20 cm into the ground (Achten *et al.*, 2008). Although Jatropha can survive precipitation as low as 300mm by shedding its leaves, it does not produce well under such conditions. An economic sustainable oil production is achieved with at least 750 mm annual rainfall or supplementary irrigation (Henning, 2007b). Intercropping of Jatropha with plants which don't share common pests and disease is recommended in the first 5 years. Spacing of 3.0 meters apart and pruning down to 65 cm can be done. Trials in Uttar Pradesh, India, found that groundnuts could be grown successfully between lines of Jatropha where the system helped in weed control of the plantation and gave better growth (Singh *et al.*, 2007).

Cultural practices in new plantations, thence, include regular weeding, pruning and fertilization. Standard management plant density is 1350-2500 plants per hectare (Henning, 2007b). Pruning is very important as it determines seed yield and can facilitate manual and mechanical harvesting of fruits. It should be done when the tree sheds leaves and enters a period of dormancy (Kaushik *et al.*, 2007), that is usually coinciding with the dry season. Canopy size determines the maximum number of

flowering branches. Large trees on a low planting density or smaller plants on high densities can apparently both results in sufficient flowering branches (Daey *et al.,* 2007).

Many people belief that Jatropha is not prone to pests and diseases which to some extent has caused economic damage. Jatropha is susceptible to numerous pest and diseases (Heller 1996; Grimm and Maes 1997). Economic damage in continuous Jatropha monocultures in India has already been observed (Shanker and Dhyani 2006). Some of the major pests are *Scutellera nobilis* bug and the inflorescence and capsule-borer *Pempelia morosalis, Pachycoris klugii* and *Leptoglossus zonatus* identified in Nicaragua (Shanker and Dhyani , 2006; Grimm and Maes , 1997). It is also believed that Jatropha can transmit the cassava super elongation disease (*Sphaceloma manihoticola*) and is a possible host for African Cassava Mosaic Virus (Heller, 1996).

3.3 Materials and methods

3.3.1 Study site

Survey and collections were done in Coastal, Eastern, Nyanza, Central, Nairobi and Rift Valley regions of Kenya (Figure 3.1). This was done using geographical mapping information gathered from the National Museums of Kenya (NMK), Kenya Forestry Research Institute (KEFRI), Green Africa Foundation (GAF) and Ministry of Agriculture (MOA) documentations. Specific areas within the above regions were identified through consultations with the regions government of Kenya forestry and

agricultural officers and key extension officers knowledgeable with the regions for collection and sampling. The areas sampled were Eastern, Coast, Rift valley, Western, Nairobi and Central.

3.3.2 Field survey

A field survey was carried out to gather information on current agronomic and cultural practices on Jatropha production in Kenya between December 2008 and April 2009 in the areas in section 3.3.1.

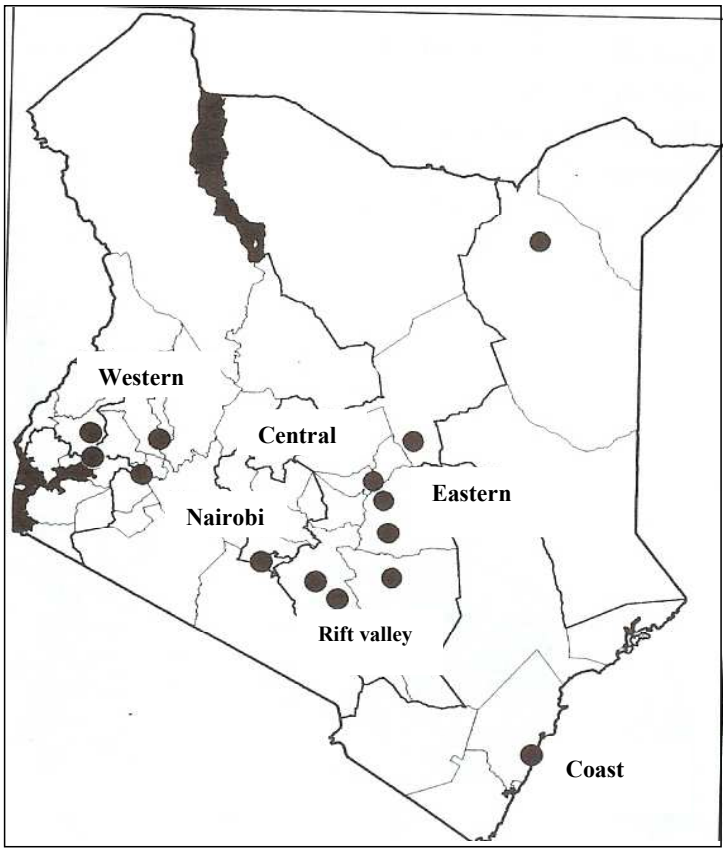


Figure 3.1: Map of Kenya showing sampled Jatropha growing regions. (Source: National Museums of Kenya, 2008).

3.3.3 Germplasm collection and management

A complete random design was used during sampling in the main growing regions. The collection of plant material was based on the availability in Coastal, Eastern, Nyanza, Central, Nairobi and Rift Valley regions of Kenya. A minimum distance of 100m between individuals was used to avoid the risk of selecting closely related individuals. Collections consisted of leaves, cuttings (Plate 3.1), seeds (Plate 3.2) and seedling (Plate 3.3). Young, clean and healthy leaves were collected in labeled polythene bags and kept in cool boxes. Mature seeds were derived from ripe fruits ensuring collection from all sides of the plant and kept in labeled paper bags. Cuttings were selected from young and healthy trees using a sharp knife. The cuttings were in sizes of 45 cm. These samples were later transferred to the Institute for Biotechnology Research (IBR) laboratory at Jomo Kenyatta University of Agriculture and Technology (JKUAT) where the leaves were stored at - 70^0C for use in molecular studies. Seeds were dried at room temperature where some were germinated on sand and others stored in paper bags in a cool well ventilated area in the laboratory. The seedlings and cuttings were transplanted in potting bags containing well mixed forest soil, sand and manure in the ratio of 2:1:1 and kept in the IBR green house. They were watered thrice a week using a spraying can to a maximum of 1 litre per pot. Germinated seeds were transplanted into potting bags as described above. Well adapted seedlings were used in subsequent experiments. All the collected germplasm were assigned a code number.

Plate 3.1: a.) Collecting of Jatropha cuttings at Tharaka b.) The Jatropha cuttings ready for propagation in IBR green house JKUAT

Plate 3.2: a) Jatropha fruits collected at G.A. F, Kitui b.) The collected seeds at IBR Lab, JKUAT

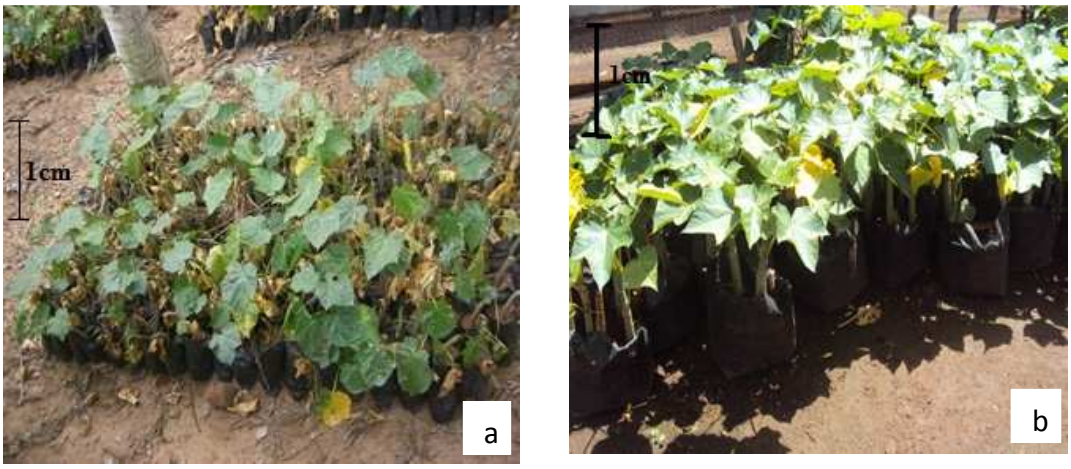


Plate 3.3: Jatropha seedlings collected at G.A.F., Kitui nursery b.) The transplanted seedlings in IBR greenhouse, JKUAT

3.3.4 Agronomic and cultural practices

Structured questionnaire (Appendix 1) was given to 100 Jatropha growers, 50 farmers' interviews were carried out and personal observations noted. Data on the agronomic and cultural practices included source of planting material, production constraints and means of propagation. Other information gathered included Jatropha uses, yielding frequency, occurrence, age, height, soil type, topography and habitat.

3.3.5 Data analysis

Data collected was presented in *MS* excel spreadsheets and analyzed using Genstat 12th Edition and Statistical Analysis System (SAS) statistical software version 9.1. The distribution and production characteristics percentage was calculated, statistical significant difference in agronomic and cultural practices and the constraints to Jatropha production in Kenya was assessed by analysis of variance (ANOVA), the data were subjected to Duncan multiple range.

3.4 Results

3.4.1 Collection and distribution of Jatropha germplasm in Kenya

During the field survey use of local names by communities was reported. Coastal region had the highest number of local names in use; Maumba, Mkina, Mboni Koma, Mibono Koma and mudiseli followed by Eastern Kyaiki kya kyeni, Kyaiki kya muunyi and Mwariki, in Western Jok while in Central and Nairobi use of the common name Jatropha was recorded. The names were mainly derived from the species use (Table 3.1).

Table 3.1: Region, counties and local names of the Jatropha accessions studied

Province	County	Local name	Use
Rift valley	Kajiado	Mchunga Kaburi	Fence, grave, oil
Eastern	Kitui	Kyaiki kya kyeni, Kyaiki kya muunyi	Fence, oil
Eastern	Makueni	Kyaiki kya kyeni, Kyaiki kya muunyi	Fence, oil
Eastern	Meru	Mwariki	Fence, oil
Eastern	Tharaka Nithi	Mwariki	Fence, oil
Coast	Taita Taveta	Mboni Koma	Fence, medicine, oil
Coast	Kwale	Mkina, Maumba, Mudiseli	Fence, medicine, oil
Coast	Kilifi	Mibono Koma	Fence, medicine, oil
Western	Kisumu	Jok	oil
Nairobi	Nairobi	Jatropha	oil
Central	Kiambu	Jatropha	oil

A total of 96 Jatropha accessions were collected with 42 accessions from Eastern, 16 Rift Valley, 14 central, 7 Nairobi, 7 Coast and 10 Western provinces of Kenya (Table 3.2). The occurrence of Jatropha accessions varied from one region to another. Kitui, Meru and Thika reported high occurrence followed by Magadi while Malindi had the lowest. In total Eastern and Rift valley provinces had the highest number.

Jatropha was found to be growing in different ecologies ranging from reserve, bush, markets, farm and fence (Table 3.2). Fence was the highest habitat type (38.5 %) and bushland (2.0 %) was the lowest, while farm (35.4 %), reserve (15.6 %) had an average score (Table 3.3). The plants were found growing in loam and clay in both flat and sloppy terrain. During the survey accessions were observed to occur either as individuals (39.5 %) or as a group (60.5 %). Two types of genetic status were reported, landrace (89.4 %) and wild type (10.6 %). Their age varied from less than 1 year to 45 years and a height of less than 1 to 8 m was reported depending on age. Jatropha was reported to be used for different purposes; 71 % hedge, 15 % medicine, 10 % biodiesel production and 4.0 % conservation. Jatropha faired on different soil textures with 71 % accessions growing on loam, 18.5 % on clay and 10.5 % on sandy clay. All farmers propagated their plants by seeds while 64.2 % of them used cuttings. Farmers reported different yielding intervals of once to twice a year or even throughout the year depending on the soil fertility and availability of water. There was continuous fruit production where the soils were fertile and also there was rain through out the year like in Malindi. Pests and diseases were reported as the major production constraints followed by low rains (Table 3.3).

Table 3.2: Documentation of various production characteristics and aspects of Jatropha in all sampled areas

Trait	Type	%
Soil type	loam	71.0
	clay	35.6
Topography	Flat	60.5
	Sloppy	39.5
Plant Age	0-5 yrs	62.0
	5-20 yrs	28.0
	20-40 yrs	6.5
	Above 40 yrs	3.5
Plant Height (m)	›1.5	71.0
	‹1.5	29.0
Occurrence	Group	60.5
	Individual	39.5
Habitat	Fence	38.5
	Farm	35.4
	Reserve	15.6
	Market	5.2
	River	3.1
	Bush land	2.0
Planting material	Seed	100.0
	Cutting	64.2
Yielding	Once	79.2
	Twice	10.4
	Throughout	10.4
Constraints	Diseases and Pests	74.2
	Rains	25.8
Uses	Biofuel	10.0
	Medicine	15.0
	Fence	71.0
	Conservation	4.0

3.4.2 Field preparation

Cultural and agronomic practices were reported in Eastern (Kitui, and Kibwezi), Central (Thika) and Coast (Malindi) by few farmers while no documentation was done

in Western, Nairobi and Rift valley. Majority of farmers had Jatropha as a hedge due to its toxicity whereby it was not fed by animals hence a good live fence material. Preparation of holes was done before the onset of the rains with a spacing of 2.0 - 2.5 m within rows and 3.0 m between rows giving a total of 2000 plants per hectare (Plate 3.4). For hedgerows it was an approximation of 15 - 25cm a part (Plate 3.5). Land tilling was by animal power through ploughing. Contours were ploughed between several rows to control soil erosion (Plate 3.6).

Table 3.3: Cultural practices for Jatropha in sampled districts

Province /district	Hole preparation	Land tillage	Contour ploughing
	(No. of farmers per district in %)		
Eastern/Kibwezi	33.3	33.3	0
Eastern /Kitui	100	100	66.7
Coast /Malindi	33.3	33.3	0
Central /Thika	66.67	66.67	33.3

Plate 3.4: Between and within rows spacing in a farm at Kibwezi district, Kibwezi location.

Plate 3.5: Row spacing in a homestead fence in Mtombo location, Tharaka district.

Plate 3.6: Contour ploughing practice in Jatropha farm in Vanilla Africa Foundation, Kitui district.

3.4.3 Jatropha farm management

Agronomic practices were reported in several districts including Malindi, Kibwezi, Meru, Tharaka, Magadi, Namanga, Thika and Kitui. High documentation of management practices was reported in Kitui and Thika while Meru and Tharaka had the lowest. These practices included pest and disease management, intercropping, irrigation, weeding and pruning (Table 3. 5). Pruning was highly practiced on the fence to encourage lateral growth in 6 districts. Pest and disease management was done at low rates due to lack of knowledge on which pesticides to use. There were no reports of either farm yard manure or fertilizer use by farmers due to claims that Jatropha can survive in less fertile soils. Majority of the farmers depended on rain fed agriculture with a few practicing irrigation and the rest left the plants to survive till the next rain season. Weeding was done when necessary, intercropping of Jatropha with other plants like kales, tomatoes and cedar was reported (Plates 3.7 and 3.8). Farmers in Thika and Kitui districts were reported to carry out both pest and disease management using Redomil, Duduthrin and Dynamec.

Table 3.4: Agronomic practices in sampled areas.

Province	District	Weeding	Irrigation	*P and D	Pruning	*I.C
Eastern	Kibwezi	66.6^{a}	33.3 ^{b}	0^{c}	0^{c}	0^{c}
Eastern	Kitui	100^{a}	100^{a}	33.3^{c}	66.6^{b}	100^{a}
Eastern	Meru	0^{b}	0^{b}	0^{b}	100^{a}	0^{b}
Eastern	Tharaka	0^{b}	0^{b}	0^{b}	100^{a}	0^{b}
Rift valley	Magadi	0^{c}	33.3 ^{b}	0^{c}	100^{a}	0^{c}
Rift valley	Namanga	66.6^{a}	0^{b}	0^{b}	66.6^{a}	66.6^{a}
Coast	Malindi	33.3^{a}	33.3^{a}	0^{b}	0^{b}	0^{b}
Central	Thika	100^{a}	100^{a}	100^{a}	33.3^{b}	0^{c}

Means with the same letter within the same row are not significantly different using Duncan multiple range test ($P_{0.05}$). ***P and D** – Pests and diseases. ***I.C** – Intercropping.

Plate 3.7: Kales intercropped with Jatropha in Kitui district, Green Africa Foundation.

Plate 3.8: Cedar and tomatoes intercropped with Jatropha in Kibwezi district, Matiliku location.

3.4.4 Propagation of Jatropha

The two methods of propagating Jatropha reported during the survey were the use of cuttings (Plate 3.9) and seeds (Plate 3.10). All farmers were reported to use seeds while 64.2 % of them also used cuttings (Table 3.6). None of the farmers reported direct sowing, 100% used nursery raised plants. The best time for transplanting was at the onset of the rains.

Table 3.5: Jatropha propagation materials in the sampled regions

Province	District of collection	Planting material	*Source
Coast	Kilifi	seed	unknown
Eastern	Kibwezi	Seed and cuttings	Coast
Western	Kisumu	seed	unknown
Eastern	Kitui	Seed and cuttings	NGO
Coast	Kwale	Seed and cuttings	unknown
Rift valley	Magadi	Seed and cuttings	unknown
Coast	Malindi	seed	unknown
Eastern	Mbitini	Seed and cuttings	Local school
Eastern	Meru	Seed and cuttings	unknown
Coast	Msabweni	seed	Tanzania
Nairobi	Nairobi	seed	Kisumu,Coast,Thara
Rift valley	Namanga	Seed and cuttings	NGO
Eastern	Tharaka	Seed and cuttings	unknown
Central	Thika	Seed and cuttings	magadi

* The origin of the planting material

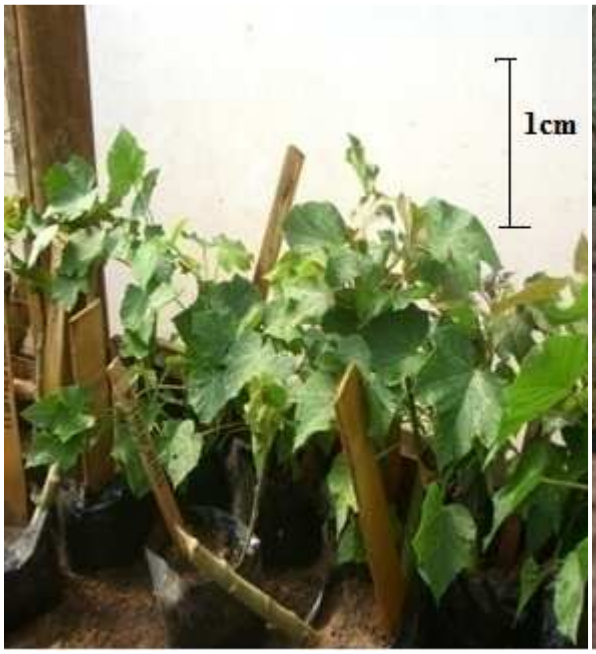


Plate 3.9: Planting material generated from cuttings in JKUAT greenhouse Thika district.

Plate 3.10: Planting material generated from seeds in Green Africa Foundation in Kitui district.

3.4.5 Constraints in Jatropha production

Jatropha production was reported to face several constraints including different diseases, management practices, inadequate water and market. Market was reported to be the highest constraint while root rot was the least. Thika district where majority of plants were on farm faced the highest challenges in production while Mbitini district where majority of the plants were hedges reported the lowest (Table 3.7).

Pests reported to be affecting Jatropha included Spider mites (Plate 3.11), whitefly (Plate 3.12) and Red beetles (Plate 3.13) while diseases were Leaf rusts (Plate 3.14), Leaf curl (Plate 3.15) and Powdery Mildew (Plate 3.16). Farmers reported use of pesticides like Dynamec and Redomil to control some of these pests and disease respectively with the majority having no control measures hence incurring economic loss.

Table 3.6: Constraints to Jatropha production reported in sampled regions.

Province	District	Mite (%)	Beetle (%)	Market (%)	Material (%)	Mildew (%)	Rust (%)	Rot (%)	*A.P (%)	Flies (%)
Eastern	Kangundo	33	33	0	0	0	0	0	0	0
Eastern	Kibwezi	0	0	100	100	0	0	0	100	0
Coast	Kilifi	0	0	100	33	0	0	0	0	0
Eastern	Kitui	0	0	100	33	33	33	0	67	0
Coast	Kwale	0	0	100	0	0	0	0	0	0
***R.V**	Magadi	0	0	100	0	0	0	0	0	0
Coast	Malindi	0	0	100	0	0	0	0	100	0
Eastern	Mbitini	0	0	0	0	0	0	0	0	0
Eastern	Meru	67	0	100	0	0	0	0	0	0
***R.V**	Namanga	0	0	100	0	0	0	0	0	0
Eastern	Tharaka	0	0	100	0	0	0	0	0	0
Central	Thika	67	33	0	100	67	33	33	0	33

*R.V – Rift valley, A.P – Agronomic practices.

Plate 3.11: Spider mites on a Jatropha flower in JKUAT green house.

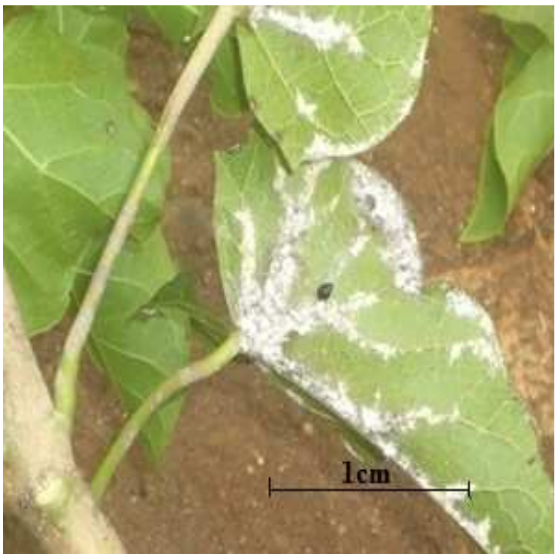


Plate 3.12: Whitefly infections on leaves in Nchiru location, Meru district.

Plate 3.13: Red beetle on stem at JKUAT farm.

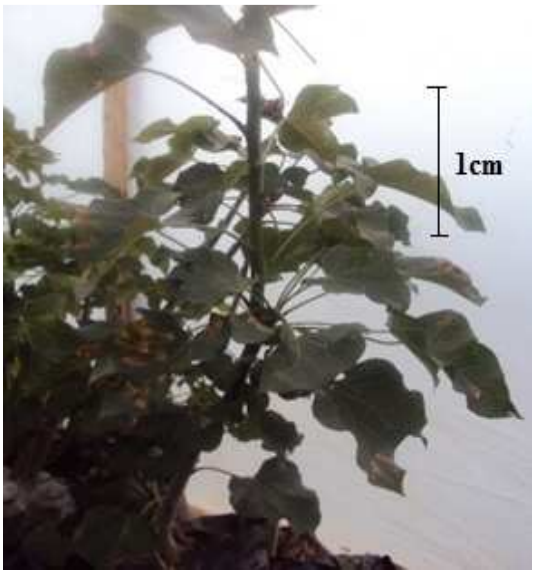


Plate 3.14: Leaf rust in JKUAT green house.

Plate 3.15: Leaf curls disease on a Jatropha plant in JKUAT green house.

Plate 3.16: Powdery mildew on Jatropha fruits in Green Africa Foundation, Kitui district.

3.5 Discussion

According to Maundu and Tengnäs, (2005), Jatropha in Kenya has been found growing in bush lands, along rivers and at homesteads as a hedge in different parts of the country in altitudes of 0-1,650 meters above sea level. This concurs with our study done in Kibwezi, Kyulu hills and in Kitui at River Mutweii where Jatropha was found growing naturally in the bush and along the river respectively. Our survey documented that most Jatropha is semi domesticated and is mainly grown as a hedge and boundary marker which is in agreement with Heller, 1996; Kumar and Sharma, 2008.

In some studies by Akintayo, 2004; Gubitz *et al.,* 1999 Jatropha is referred to as Black vomit nut", "Purge nut", "Physic nut", "poison nut tree" the "graveyard tree'' due to the toxicity of the seeds and around 200 different names for Jatropha have been

reported (Katembo and Gray, 2007). The study revealed that in Kenya several local names are used by different communities based on its uses like “Mchunga Kaburi” to mark graves, “Mudiseli” for diesel production and “Kyaiki kya muunyi” for shade. Naming was also as a result of different ethnic groups in Kenya like “Maumba”, “Mkina”, “Mboni” “Koma”, “Mibono” “Koma”, “Kyaiki” and “Mwariki” as reported by Maundu and Tengnäs, (2005).

Kitui and Magadi in the Eastern and Rift valley provinces respectively reported the highest distribution of Jatropha. This may be as a result of the existence of NGO’s in Kitui (Green Africa Foundation and Vanilla Foundation) and Ngurumani irrigation scheme in Magadi that have supported the communities in growing Jatropha for biodiesel. Introduction point in Namanga Tanzania boarder by the colonialists as reported by the community could also have led to the distribution pattern. Around 45 years old Jatropha plants were reported in Namanga and Meru which were still productive which is in agreement with reports that Jatropha has a long productive period of around 30 - 50 years (Banapurmath *et al.,* 2008; Tamalampundi *et al.,* 2008). Although some plants were reported to be 8 meters high, on average majority were 5 meters high (Heller, 1996).

Due to misleading information and lack of knowledge, both cultural and agronomic practices were carried out at very low rates compared to reports from Zimbabwe, Brazil, Mali and India where high yields have been achieved (Openshaw, 2000). In our study Jatropha was mainly found growing in semi arid regions where rains were

not reliable and no irrigation was done hence the plant was dormant for several months leading to delayed maturity and low yields. Aerated sands and loams were best suited while clays particularly with poor drainage were less suitable as reported by (Gour, 2006). Jatropha was also observed to be able to survive in very poor dry soils in conditions considered marginal for agriculture like in the Magadi Soda region. However, survival ability didn't mean that high productivity could be obtained from Jatropha under marginal agricultural environments as these plants were only 50 cm tall yet 3 years old. Few farmers enriched their soil using organic manure with majority thinking it's not necessary to enrich the soil due to the claim that Jatropha can survive in less fertile soil (Singh *et al*., 1996). Intercropping with other plants like vegetables and trees was documented in Kitui and Kibwezi at earlier years (Singh, 2007), which helped in weed control, pests and disease, push pull, compared to non intercropped Jatropha. Weeding was practiced regularly to avoid nutrient competition and prevent pests and diseases by most farmers. Pruning during the dry or dormant season was done by few farmers to maintain fence height and form a wide low-growing tree that could make a good fence (Gour, 2006). The amount of rainfall experienced in most Jatropha growing regions was only enough for Jatropha survival while for it to produce at least 600 mm (FACT, 2007) was necessary. Reports from earlier geographical mapping by KEFRI and NMK showed that no Jatropha was growing on the Kenyan highlands which are characterized by higher precipitation which corresponds to (Foidl, 1996) and (Achten, 2008), that Jatropha has not been found growing in more humid areas of origin, Central America and Mexico. Kilifi County

reported continuous flowering throughout the year in areas with continuous rainfall. Propagation was mainly through seeds with few farmers using cuttings, information on the source of planting material and plant raising requirements was not available to farmers unlike in India where superior provenances with high yield and oil content had been used (Kureel, 2006).

The selection of planting material was based on what was available and not from cuttings or seed that had been proven over several seasons to have high yield and seed oil content as proposed for starting a new plantation. This was contrary to Achten, (2008), that trees capable of producing more than 2 tonnes of dry seed per hectare with 30 percent seed oil content should be selected as the source of planting material. Due to lack of knowledge that seed raised plants had a higher survival rate, longer lifespan as a result of taproot presence compared to plants raised through cuttings although the later matured early (Henning, 2009), farmers still used either. Farmers also did not take into consideration use of specific diameter or length of cutting as reported by Heller, (1996) of 30 mm diameter and 30 cm length. Plants raised through cuttings seemed weak due to lack of a taproot meaning poor soil anchorage, less capacity to extract water and nutrients, less suitability to intercropping and shorter productive life of the plantation hence larger cuttings are recommended for maximum production. Use of tissue culture planting material was not reported since they were not available to farmers. Plant densities of seedlings ranged from 1800 – 2300 per ha in a spacing of 2.5 x 3.0 to 3.0 x 2.0 meters. This was close to that by Achten (2008) of 3.0 x 2.0, 3.0 x 2.5 or 3.0 x 3.0 meters. No farmer observed width and depth of 30 - 45 cm and

no insecticide use as a precaution against termites was reported. For cuttings, spacing between 15 - 25 cm close to Achten's (2008) of 5 and 25 cm apart was practiced.

Farmers had several production constraints like lack of market for their produce, pests and disease attacks, lack of quality planting material, lack of knowledge on best management practices and low rains. Although few farmers had seeds ready for sale, 75 % feared that their produce will not have ready market since there was no actual production of Jatropha oil in Kenya unlike other countries like India where sale price of Jatropha biodiesel was around US$ 0.68 per litre (Tewari, 2007).

Fungal diseases such as powdery mildew were observed on the underside of the leaves where it developed typical powdery and white growth. Other diseases like leaf rust and leaf curl were found in some of the farms sampled. These caused discoloration, leaf folding and leaf falling, reducing rate of photosynthesis hence slow growth rate and low fruit yields. Red beetles were the most destructive pests of Jatropha especially in Thika district. They sucked the leaf fluid which eventually dried up and fell. Spider mites were also found covering Jatropha leaves which turned brown and dropped prematurely. Wet and cloudy weather was found to favour disease infections as reported by Gour *et al.,* (2008) while dry spell favoured pests' infections as a result of reduced number of prey. Control of these diseases and pests was by use of available pesticides in the market and biological practices like timely weeding. These findings confirm earlier reports (Daey Ouwens *et al.,* 2007) that the plant is vulnerable to most

common pests and diseases found in food crops hence methods of control can be shared. Intercropping with plants which are alternate hosts should be controlled.

3.6 Conclusion

Total Jatropha accession collected were 96 in form of seeds, seedlings, cuttings and leaves. No commercial cultivation of Jatropha was documented and neither of those documented was reported to be improved varieties. Jatropha accessions were identified by farmers in their ethnic languages according to their phenotypic character or use. They were reportedly grown mainly by farmers as a fence and few on farm by small scale farmers. The cultivated Jatropha had high pests and disease infections while the wild and semi cultivated showed low disease incidences may be due to alternative hosts. Propagation was done through seeds and cuttings and no tissue culture planting material was reported. Farmers showed lack of considerable knowledge about Jatropha crop management and lacked quality planting material. There was no remarkable morphological difference noted on Jatropha collected all over the country which could lead to different varieties hence need for genetic diversity studies.

3.7 Recommendations

Farmers need to be trained on agronomic and cultural practices to increase production. Plantation should be started with locally available varieties which have shown low disease and pest incidences and high production and not with unknown varieties unless they are confirmed to be viable. Research on the best cropping system for Jatropha and training farmers on Jatropha field management practices need to be done as one of the activities geared towards improvement of its production in Kenya. Research geared towards improving the quality and increasing the quantity of planting material should be done. These can be done by investigating further, the pests and diseases affecting Jatropha, black listing accessions with quantity and quality traits, then improvement through breeding and genetic engineering.

CHAPTER FOUR

4.0 DEVELOPMENT OF AN *IN VITRO* REGENERATION PROTOCOL *VIA* SOMATIC EMBRYOGENESIS FOR SELECTED KENYAN JATROPHA ACCESSIONS

4.1 Abstract

Jatropha curcas L. is peculiar treasured tree species for its uses and considerable economic potential as a biofuel plant. Propagation of Jatropha is mainly done through seeds and cuttings. Propagation using seeds is limited by low viability and germination hence unable to provide enough planting material. This is a major constraint in Jatropha production in the major growing regions of Kenya. In this study five Jatropha accessions JRV1, JCO4, JE4, JN1 and JNY1 each representing the major Jatropha growing regions in Kenya were selected randomly and used in the development of an *In vitro* regeneration protocol.

Somatic embryogenesis was applied involving sterilization, initiation, callus induction, embryo formation, shoot proliferation, root development and hardening in the greenhouse. Young leaf discs and petioles explants were cultured on Murashige and Skoog (MS) medium supplemented with 0.5 - 2.0 mg/l 6- benzylaminopurine (BAP) 0.4 - 0.7 mg/l Kinetin (KIN) , 0.3 - 0.6 mg/l Indole -3-acetic acid (IAA) and 0.1 mg/l Thiadiazuron (TDZ) . Effect of plant growth regulators was tested on callus induction, embryogenic callus development, shoot proliferation and root induction. All treatments were laid out in a complete randomized design.

Analysis of variance was performed and significant differences among treatment means calculated ($P < 0.05$). Explants were best sterilized with 20% Sodium

hypochlorite for 20 min, JNY1 leaf discs and petioles had the highest survival rate (88.9%). MS medium supplemented with 1.5 mg/l BAP, 0.6 mg/l KIN, 0.3 mg/l IAA and 0.1 mg/l TDZ induced embryogenic calli across all accessions tested with JN1 accessions having the highest frequency of 85%. Embryo development showed high frequency of shoot formation (8.25 cm) in JN1 accessions. MS supplemented with 3.5 mg/l indole-3-butyric acid (IBA) and 3.5 mg/l Naphthalene acetic acid (NAA) gave an average root formation of 2.5 cm on JCO4, JN1, JE4 and JRV1 accessions. The rooted plants were established in forest soil, sand and manure mixed in the ratio of 2: 1:1 in the green house with 20% survival rate. This successful *in vitro* regeneration is vital for production of quality and quantity planting material.

4.2 Introduction

The genus *Jatropha*, which is perceived to comprise approximately 170 known species, is distributed in the tropical and subtropical world. Jatropha has been considered as a plant for biofuel production mainly due to its high seed oil content of 40-50% and non-competing demand with edible oil supplies (Rajore *et al.,* 2007). With the recent increase in fuel prices there is an increased demand in the use of Jatropha oil to alleviate energy crisis. Conversion of the oil to biodiesel is relatively simple by chemical (Berchmans and Hirata 2008) or biological trans-esterification (Modi *et al.,* 2007). Jatropha is also desired due to its drought tolerance, rapid growth, low cost of seeds, oil content and short maturity period with wide range of environmental adoption (Gubitz *et al.,* 1999; Jones *et al.,* 1992). The oil is also used in

manufacturing candle, varnishes, soap and treatment of several diseases among others uses (Rajore *et al.,* 2007). Although Jatropha has assumed paramount importance as a potential biodiesel crop in more than 50 countries, its production is met with limited success. This is due to unreliable seed production, low oil yields, vulnerability to pests and diseases and low economic returns (Heller, 1996; Kaushik *et al.,* 2007).

Commercial propagation of Jatropha is typically through seed and vegetative cutting. Vegetative propagation of Jatropha through stem cuttings has been achieved however the established plants are not deep rooted and hence, they easily get uprooted when cultivated in lands with poor top soil (Heller, 1996; Openshaw, 2000). Despite their profuse vegetative growth, the number of seeds produced per plant is very low and the seeds show a low seed fecundity, which is reduced by 50% within 15 months. Plants propagated by cuttings show a lower longevity and possess a lower drought and disease resistance than those propagated by seeds (Sujatha *et al.,* 2007). Traits like higher seed yield, oil content, synchronous maturity and early flowering can be introduced for production to be sustainable and viable. Application of plant biotechnological methods can improve the crop by producing disease free plants, large number of plantlets and conserve the germplasm. Plant species with rich secondary metabolites like Jatropha have proved to be difficult for mass propagation through tissue culture. Development of an efficient *In vitro* regeneration system would be a remarkable progress for the Jatropha business and the field of alternative energy technology (Rajore *et al.,* 2007).

Somatic embryogenesis and organogenesis using different explants have been reported in Jatropha (Sujatha *et al.,* 2008; Rajore *et al.,* 2007; Kalimuthu *et al.,* 2007). Low rates of *In* vitro multiplications were reported in these studies. Somatic embryogenesis is a process by which somatic cells or tissues develop into differentiated embryos. It is the gateway to efficient propagation and genetic transformation due to its single cell origin. Evaluation of tissue culture propagated plants of Jatropha revealed that they were at par with seed propagated plants in terms of yield and yield related traits (Sujatha *et al.,* 1996). There are no documented studies on micropropagation of Kenyan Jatropha genotypes which can be used for mass production. Moreover, no field transfers of Jatropha tissue culture plantlets have been successfully reported in previous studies. Rooting *In vitro* and acclimatization have also proven very difficult for Jatropha (Pankaj *et al.,* 2011). Therefore, the objective of this study was to develop an reproducible *In vitro* regeneration system for mass micropropagation of Kenyan Jatropha genotypes.

4.3 Materials and methods

4.3.1 Plant material

The study was carried out between December 2008 and October 2010 at the IBR laboratories in JKUAT. Plant materials used in this study were collected between December 2008 to April 2009 from the Coastal, Eastern, Western, Central, Nairobi and Rift Valley provinces of Kenya (Section 3.4.1, Table 3.2). The plant materials collected consisted of mature seeds, cuttings and seedlings. Seeds and cuttings were

collected from mature and healthy plants whereas the seedlings were less than six months old. At least 10 fruits were harvested, 5 cuttings and 5 seedlings collected per plant. They were stored in cool boxes and later transported to IBR, JKUAT and planted as described in section 3.3.3. The seeds were germinated on growth trays containing sand and kept in the same greenhouse. They were watered thrice a week using a spraying can. Seed that germinated were transplanted into potting bags containing soil, sand and manure in the ratio of 2:1:1 after two weeks. Five accessions (Namanga, Markebuni, Tharaka, Museum and Kisumu) o f well adapted plantlets were used in subsequent experiments (Table 4.1).

Table 4.1: Jatropha accessions used in regeneration

Province	Collection point	Accession code
Rift valley	Namanga	JRV1
Coast	Markebuni	JCO4
Eastern	Tharaka	JE4
Nairobi	Museum	JN1
Western	Kisumu	JNY1

4.3.2 Chemicals and culture conditions

Murashige and Skoog (MS), basal medium (Murashige and Skoog, 1962) used contained different constituents **(**Stock solution I: mineral salts, Stock solution II: vitamins and Stock solution III: iron) in grams per litre (Appendix II). All basal media

were supplemented with 3% (w/v) sucrose, plant growth regulators (PGRs) as per growth stage and the pH was adjusted to 5.8 using 0.1N HCL or 0.1N NaOH, 0.28 % (w/v) Gelrite was added and the media dispensed in 200ml culture jam jars (20ml of medium per jar).The media, water, glassware and metallic equipment used were sterilized by autoclaving at 121ºC at 15 lbs pressure for 20 min. The sterilized media was kept at room temperature for three stays before culture.

Chemicals used were of analytical grade (Qualigens, Duchefa and Sigma) and experiments were carried out under aseptic conditions in the laminar flow (Clean Bench, HITACHI Ltd Tokyo, Japan). Forceps and scalpels were sterilized in a Bead Sterilizer (Keller AG, Switzerland) at 250°C before use. To minimize contamination, precautions were taken to avoid the forceps touching the agar medium. Cultures were maintained at 25 ± 2° C under a 16 hr light and 8 hr dark period in air conditioned growth chambers illuminated by 40W provided by Philips white fluorescent tubes. The intensity of light was regulated between 2500-3000 lux.

4.3.3 Establishment of sterilization protocol

Leaf discs and petiole explants were obtained from the 3rd and 4th leaves foliar of 3 - 4 months old mother plants raised in the greenhouse (section 4.3.1). They were placed in glass beakers and kept in running tap water for 30 min to remove physical impurities and latex. They were then transferred to new glass beakers containing 250ml of water containing 100µl/l Tween®20 (wetting agent) and 2ml/l of Dettol detergent had been added. The beaker was swirled gently at intervals for fifteen minutes and leaves were

rinsed 3 times with distilled water. Thereafter the explants were kept in 0.3% (w/v) Redomil® (fungicide) plus 100µl/l Tween®20 for 1 hr then washed thoroughly with double distilled water. Under a clean Lamina flow hood, the explants were subjected to 70% (v/v) ethanol for 30 sec, rinsed with double distilled water thrice to remove all the ethanol and then subjected to 20% (v/v) sodium hypochlorite containing 100µl/l Tween®20 at varying exposure times; 15 min, 20 min and 25 min then rinsed three times with double distilled water. The leaf disc was put on initiation media with abaxial surface in contact with media while petioles explants were placed horizontally. Daily assessment and monitoring for fungal and bacterial infections of cultures was done. Data on type/rate of contamination, mortality and survival was recorded after 5 days of initiation where explants exhibiting symptoms of fungal or bacterial or both contaminations, dead or alive were scored as 1 while absence of the parameters were scored as 0. Explants were maintained on initiation media for 1 week prior to subculture on callus inducing media.

4.3.4 Effects of Plant Growth Regulators on callus induction

Murashige and Skoog medium supplemented with different PGRs at varying concentrations was used for callus induction on five Jatropha accessions; JNI, JNYI, JCO4, JE4 and JRVI (Table 4.1). Combined PGRs at varying concentrations of A (0.5 mg/l BAP,0.4 mg/l Kin, 0.3mg/l IAA and 0.1 mg/l TDZ), B (1.0 mg/l BAP,0.5 mg/l Kin, 0.4mg/l IAA and 0.1 mg/l TDZ) , C (1.5 mg/l BAP,0.6 mg/l Kin, 0.3mg/l IAA and 0.1 mg/l TDZ), D (2.0 mg/l BAP,0.7 mg/l Kin, 0.6 mg/l IAA and 0.1mg/l TDZ)

and a control (MS media without PGRs) totaling to 25 treatments were set. Media preparation was as described in section 4.3.2 above. The percentage proportion of callus induction on leaf discs and petioles was evaluated at an interval of 2 weeks after initiation.

4.3.5 Effects of Plant Growth Regulators on somatic embryo development

The same PGRs used in section 4.3.4 were used to test the rate of somatic embryos formation from callus of JN1, JCO4, JE4 and JRVI accessions. Sub culturing after every 4 weeks was carried out. Somatic embryos formation per treatment was evaluated for 2 months after every 2 weeks. The morphology of the embryos; colour, texture and shape was also determined using eye and microscopic observation.

4.3.6 Effects of Plant Growth Regulators on shoot proliferation

MS fortified with the PGRs as in section 4.3.4 was used in shoot formation, multiplication and elongation of JN1, JCO4, JE4 and JRVI. This was done for 2 months to evaluate the regeneration rate. Induction of micro shoots and their length was evaluated at an interval of 2 weeks after sub culturing.

4.3.7 Effects of Plant Growth Regulators on root induction

Half strength MS containing 3% (w/v) sucrose, 0.28% (w/v) gelrite fortified with BAP and IAA combined at varying concentrations were used. Five experiments were set; 1.5mg/l BAP and 1.5mg/l IAA, 2.5mg/l BAP and 2.5/l IAA, 3.0mg/l BAP and 3.5mg/l IAA, 4.0 mg/l BAP and 4.0 mg/l IAA and a control (MS media without PGRs)

totaling to 25 treatments were set and used in root initiation of JN1, JCO4, JE4 and JRVI. This was done for 2 months to evaluate the regeneration rate. Induction of roots and their length was evaluated at an interval of 2 weeks after sub culturing.

4.3.8 Acclimatization

Acclimatization of the plants was done in the green house in pots containing forest soil, sand and manure at the ratio of 2:1:1.

4.3.9 Experimental design, data collection and analysis

The experiments were set up in completely randomized design (CRD). For experiments on sterilization, callus induction, shooting and rooting were repeated 3 times. Each experiment had at least 5 explants per treatment. The frequency of callus formation, expressed as percentage, was calculated as the proportion of number of explants forming callus. Observation on somatic embryo germination, shoot proliferation and root induction rate were recorded. Data was collected in MS Excel spreadsheets and analyzed using Statistical Analysis System (SAS) 9.1 and GenStat 12^{th} Edition, statistical softwares. Results were subjected to analysis of variance (ANOVA) to detect significant differences between means. Means differing significantly were determined using Tukey's test at 5% probability level.

4.4 Results

4.4.1 Sterilization protocol

Low exposure time of 15 min resulted to high rate of contamination and low rates of survival while high exposure time of 25 min resulted to high mortality rate and low rates of contamination and moderate exposure time of 20 min resulted in low rates of contamination and mortality and high survival rates at $p<0.05$.

When the exposure time of 20% sodium hypochlorite was reduced from 20 min to 15 min, contamination levels in leaf discs and petioles increased from 11% to 89% across all accessions. Higher contaminations levels were noted on petioles compared to leaf discs. Mortality levels in leaf discs and petioles reduced from 89% to 0% and 78% to 0% respectively across all the accessions when the exposure time of 25 min was reduced to 20 min. At a moderate exposure time of 20 min the survival rate in leaf discs and petioles was 67% to 89% respectively across all accessions (Table 4.2).

Table 4.2: Petioles and leaf discs contamination, mortality and survival rates of five accessions when exposed to 20% NaOCl at varying exposure times.

		Leaf discs			Petiole		
Accession	**Time (min)**	**conta (%)**	**mortality (%)**	**survival (%)**	**conta (%)**	**mortality (%)**	**survival (%)**
JCO4	15	88.9[a]	0.0 [d]	11.10 [f]	100 [a]	0.0 [d]	0.0[f]
JCO4	20	22.0[c]	11.1 [c]	66.7 [c]	22. 0 [e]	11.1 [c]	66.7 [c]

JCO4	25	0.0[d]	88.9[a]	11.23[f]	0.0[g]	77.8[b]	22.2[d]
JE1	15	66.7[b]	0.0[d]	33.3[d]	88.9[b]	0.0[d]	0.0[f]
JE1	20	22.0[c]	11.1[c]	66.7[c]	33.3[d]	0.0[d]	66.7[c]
JE1	25	0.0[d]	77.8[b]	22.0[d]	0.0[g]	77.8[b]	22.2[d]
JN1	15	77.8[a]	0.0[d]	22.2[e]	77.8[c]	0.0[d]	22.2[d]
JN1	20	22.0[c]	0.0[d]	88.9[a]	22.2[e]	0.0[d]	77.8[b]
JN1	25	0.0[d]	88.9[a]	11.1[f]	0.0[g]	77.8[b]	22.2[d]
JNY1	15	77.8[a]	0.0[d]	22.2[e]	77.8[c]	0.0[d]	22.2[d]
JNY1	20	11.1[c]	0.0[d]	88.9[a]	11.1[f]	0.0[d]	88.9[a]
JNY1	25	0.0[d]	88.9[a]	11.1[f]	0.0[g]	88.9[a]	11.1[e]
JRV1	15	88.9[a]	0.0[d]	11.1[f]	88.9[b]	0.0[d]	22.2[e]
JRV1	20	22.0[c]	0.0[d]	77.8[b]	22.2[e]	0.0[d]	77.8[b]
JRV1	25	0.0[d]	88.9[a]	11.1[f]	0.0[g]	77.8[b]	22.2[d]
L.S.D$_{0.05}$		14.7	7.03	5.43	4.65	6.06	6.62

Means with the same letter within the same column are not significantly different from each other at P ≤0.05 using Tukey's test.

4.4.2 Callus induction

Explants enlargement and swelling was observed after three days of culture. After 6 days mass of undifferentiated cells originated from petiole ends exposed to the medium whereas on leaf discs calli were formed all over the surface on all four groups of culture media used (Figures 4.1 a and b).The callus colour varied from green, whitish green, yellowish green, brownish green, white and brown. The texture was both compact and friable depending on PGRs and accession. Callus induction medium consisted of MS supplemented with BAP, KIN, IAA and TDZ combined and at varying levels. There was a significant effect of PGRs on callus induction on various accession of Jatropha. Explants cultured in absence PGRs senesced without producing

callus. Callus induction medium containing 1.5 mg/l BAP, 0.6 mg/l KIN, 0.5 mg/l IAA and 0.1 mg/l TDZ (C) induced callus significantly faster and in high rates (P ≤0.05) compared to that induced by low levels. At high levels of PGRs callus was induced within 2 weeks but with a relatively low frequency and growth rate. Induction rates of 18% JNY1, 65% JE4, 66% JCO4, 67% JRV1 and 85% JN1 were recorded (Table 4.3).

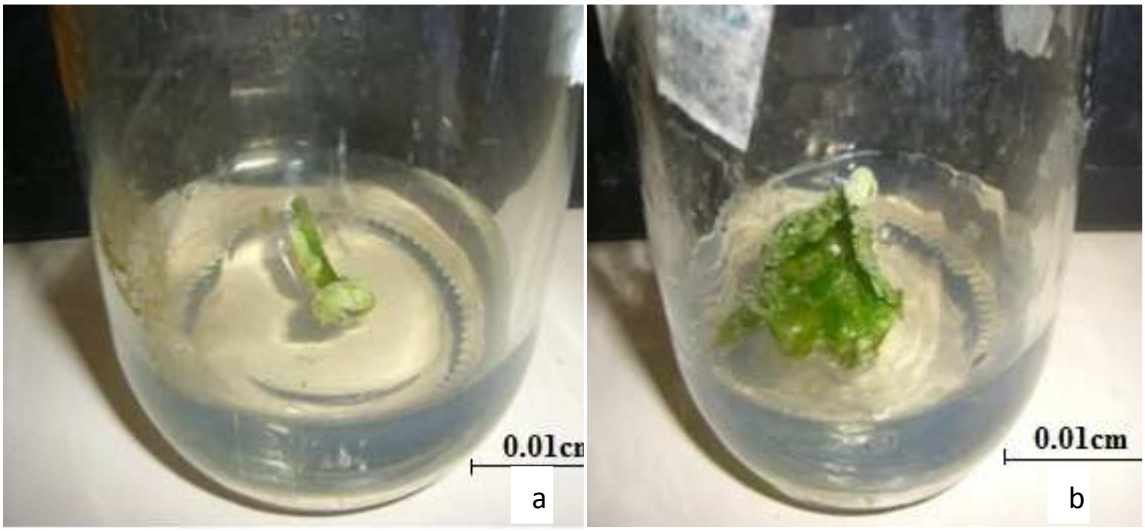


Figure 4.1: callus induction on different explants a.) Petiole explants b.) Leaf discs explants

Table 4.3: Effect of different concentration and combination of BAP, Kinetin, IAA and TDZ on % callus induction from petioles.

Accessions	Plant growth regulators (mgl^{-1})			
	* A	* B	* C	* D
JNY1	8.75±1.25^{d}	15.00±2.04 ^{d}	17.50±2.50^{c}	11.25±1.25^{b}
JCO4	46.25±2.39^{b}	55.00±2.04^{c}	66.25±2.39^{b}	52.25±2.39^{a}
JE4	15.00±2.04 ^{c}	50.00±2.04 ^{c}	65.00±2.04^{b}	50.00±2.04^{a}

JN1	8.75±1.25[d]	75.00±2.04[a]	85.00±2.04[a]	17.50±2.50[b]
JRV1	57.50±3.23[a]	65.00±2.04[b]	67.50±3.23[b]	50.00±2.04[a]

Means (± SE) followed by different alphabets in each column were significantly different at P ≤0.05 using Tukey's test.

*A = (0.5 mg/l BAP, 0.4 mg/l Kin, 0.3mg/l IAA and 0.1 mg/l TDZ)
*B = (1.0 mg/l BAP, 0.5 mg/l Kin, 0.4mg/l IAA and 0.1 mg/l TDZ)
*C= (1.5 mg/l BAP, 0.6 mg/l Kin, 0.3mg/l IAA and 0.1 mg/l TDZ)
*D= (2.0 mg/l BAP, 0.7 mg/l Kin, 0.6 mg/l IAA and 0.1mg/l TDZ

4.4.3 Embryo development and germination

After 8 weeks of culture on different concentrations, JNY1 callus produced few whitish somatic embryos some of them became necrotic. Necrosis began on the periphery and eventually the whole plant. Callus from other accession remained green, whitish green, light green, white and brown in different concentrations and retained high embryogenic potential. During embryo development friable green and compact green callus were observed to undergo globular stage, heart stage, torpedo stage and maturity stage (Figures 4.2 a, b, c and d). White and brown callus did not generate any embryos but died after subculturing. Shoots were formed on different accessions at varied rates with the lowest being 1.25 and highest 8.25.

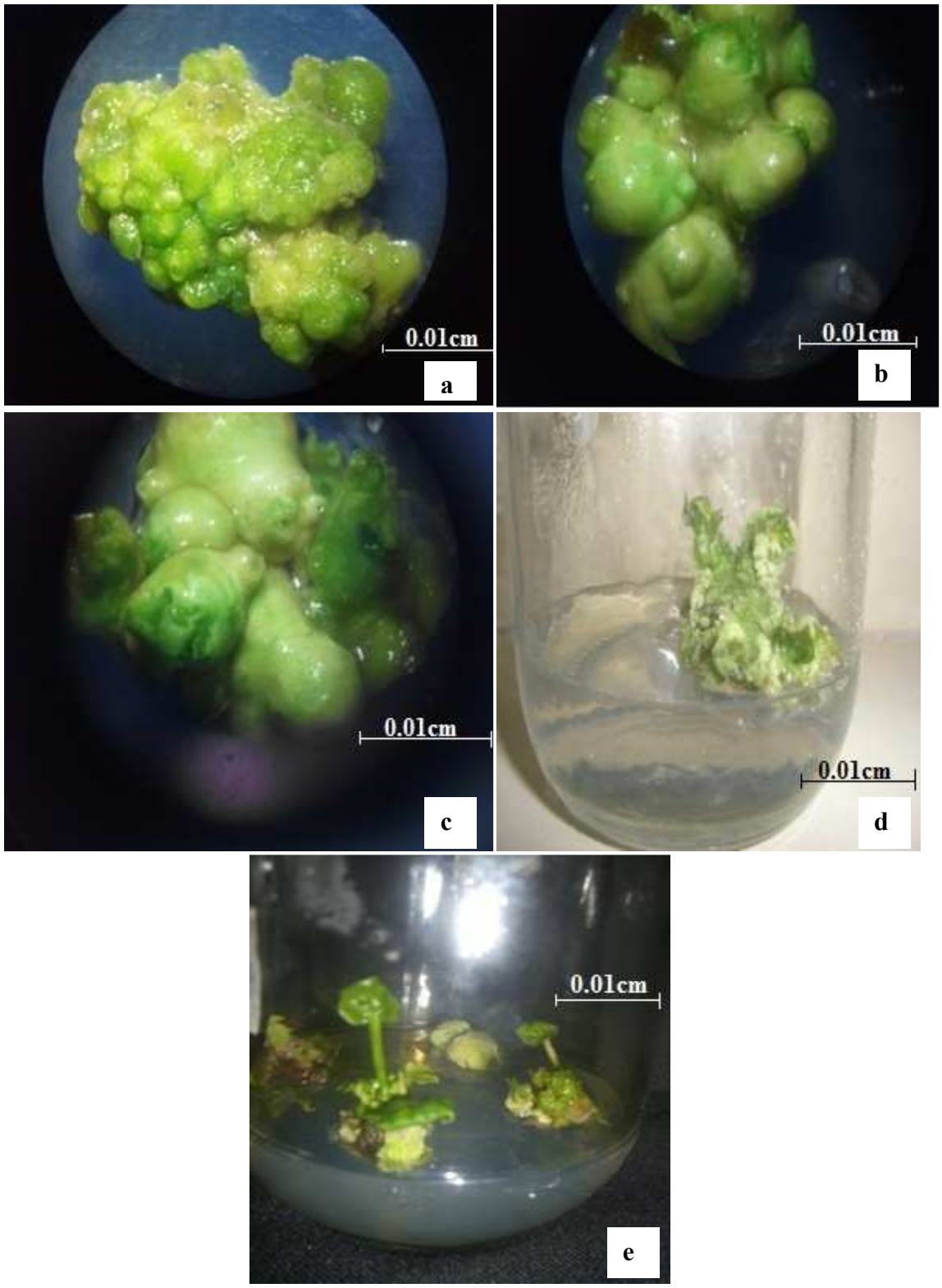


Figure 4.2: Different stages of embryo development a) globular shaped b) heart shaped c) Torpedo shaped d and e) leaf bud formation

4.4.4 Shoot multiplication and elongation media

After transfer of embryos to shoot induction media (SIM), a mean number of shoots were formed on each explant which varied significantly. The generated plants showed true Jatropha morphology. Shoot length of 1.25 cm to 4.75 cm was observed (Table 4.4). The best concentration was 1.5 mg/l BAP, 0.6 mg/l Kin, 0.3mg/l IAA and 0.1 mg/l TDZ across all the accession.

Table 4.4: Effect of different concentration and combination of BAP, Kinetin, IAA and TDZ on shoots elongation (cm).

Accessions	**Plant growth regulators (mgl^{-1})**			
	***A**	***B**	***C**	***D**
JCO4	1.25[b]	2.75[c]	4.75[c]	3.00[b]
JE4	2.00[b]	2.50[c]	4.50[c]	5.00[a]
JN1	1.25[b]	4.25[b]	8.25[a]	6.00[a]
JRV1	5.00[a]	6.25[a]	6.50[b]	5.50[a]
$L.S.D_{0.05}$	0.832	1.01	1.371	1.177

Means (± SE) followed by same alphabets in each column were not significantly different at $P \leq 0.05$ using Tukey's test.

*A = (0.5 mg/l BAP, 0.4 mg/l Kin, 0.3mg/l IAA and 0.1 mg/l TDZ)
*B = (1.0 mg/l BAP, 0.5 mg/l Kin, 0.4mg/l IAA and 0.1 mg/l TDZ)
*C= (1.5 mg/l BAP, 0.6 mg/l Kin, 0.3mg/l IAA and 0.1 mg/l TDZ)
*D= (2.0 mg/l BAP, 0.7 mg/l Kin, 0.6 mg/l IAA and 0.1mg/l TDZ

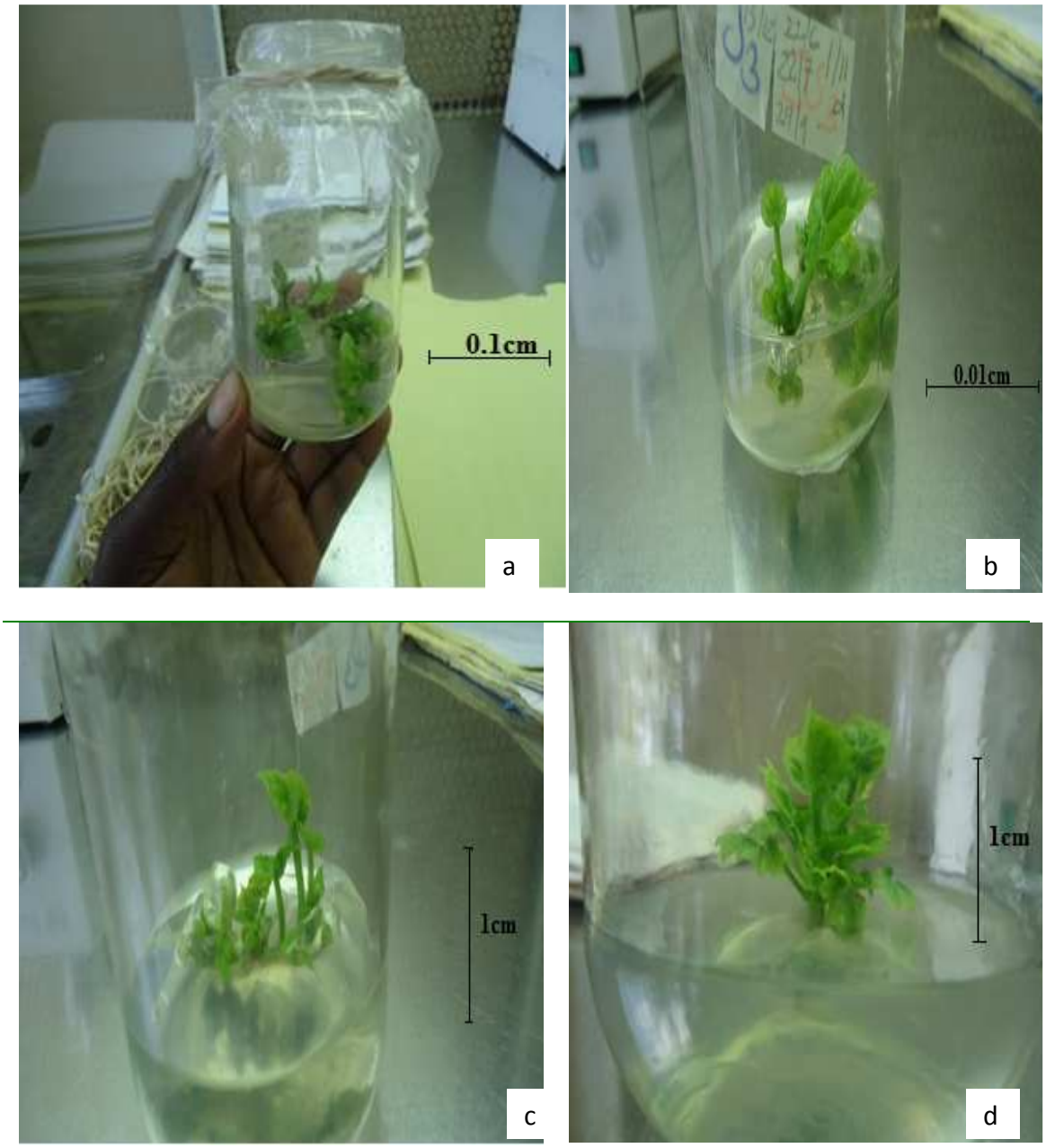


Figure 4.3: shoot formation from embryogenic callus of JN1 accessions on shooting medium a) shoot formation (2 weeks), b) shoot elongation (4 weeks), c) multiple shoot proliferation after 6 weeks, d) individual elongated shoot after 8 weeks

4.4.5 Root induction

Root induction and development required approximately 4 weeks with significant treatment effects on the rate of rooting (Table 4.5). Root development was induced in the shoot cultured on ½ MS supplemented with IBA and NAA for all accessions. No rooting occurred in the absence of auxins. Generally these treatments resulted in the formation of mean number of roots regardless of the accessions types with concentrations 3.0 mg/l IBA and 3.5 mg/l NAA being the most effective for root induction. The lowest mean root number was 1.81 while the highest was 3.50.

Table 4.5: Effects of auxins at different concentrations and combination on number of roots in excised shoots of various *Jatropha curcas* accessions.

Accessions	**Plant growth regulators (mgl^{-1})**			
	1.5 IBA +1.5 NAA	**2.5IBA +2.5 NAA**	**3.0IBA + 3.5NAA**	**4.0IBA + 4.0NAA**
JCO4	1.813± 0.120[a]	2.36±0.152 [a]	3.36±0.240 [a]	2.36±0.152 [a]
JE4	1.813±0.120 [a]	2.29±0.109 [a]	3.50±0.204 [a]	2.38±0.125 [a]
JN1	1.938±0.213 [a]	2.54±0.107 [a]	3.50±0.204 [a]	2.56±0.157 [a]
JRV1	1.06±0.329 [a]	2.24±0.165 [a]	3.50±0.354 [a]	2.36±0.152 [a]
$L.S.D_{0.05}$	0.658	0.4178	0.794	0.4531

Means (± SE) followed by same alphabets in each column were not significantly different at $P \leq 0.05$ using Tukeys test.

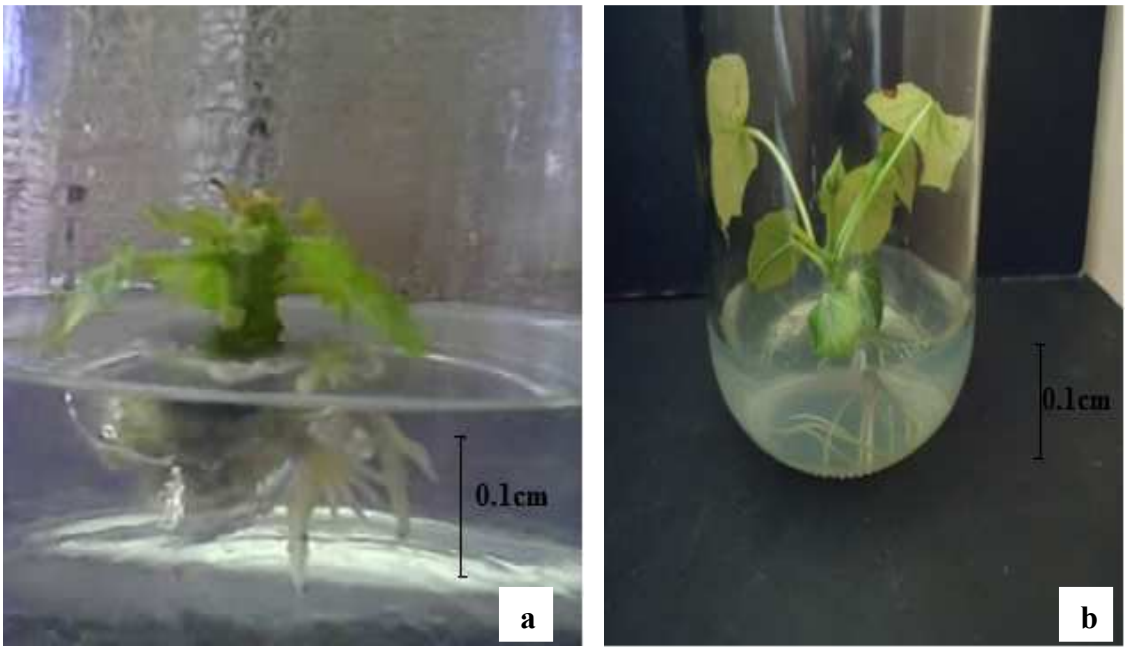


Figure 4.4: Root formation on JN1 accessions a) 4 week old short rooted plantlet b) 4 week old long rooted plantlet

Figure 4.5: JN1 accession plantlet undergoing acclimatization in pots containing forest soil, sand and manure in the ratio of 2: 1:1 in the green house.

4.5 Discussion

Somatic embryogenesis enables production of large number of plants throughout the year and is also a powerful tool for genetic improvement of all plant species as a result of its single cell origin (Bhansali *et al.,* (1991). In this study a reproducible regeneration system of Jatropha through somatic embryogenesis was developed. The plant growth regulator concentration, source of explants and genotype significantly influenced the regeneration response, as observed by Kumar *et al.,* (2010). In this study sterilization using mercuric chloride was replaced by Sodium hypochlorite, due its harmful environmental effects (Ahmad *et al.,* 2011). It was found that the optimum exposure time of surface sterilization using 20% Sodium hypochlorite was 20 min for both leaf discs and petioles in all accessions. Significant ($P<0.05$) low levels of contamination (22) and mortality (0) accompanied by high levels of survival (88.9) were reported at 20min. High levels of contaminations (100) and mortality (88.9) were observed at 15 min and 25 min respectively. Young leaf disc and petiole explants from the 3rd and 4th leaves of the five Jatropha accessions; JNI, JNY1, JCO4, JRV1 and JE4 cultured on different media formed callus. The 1st and 2nd leaves were too tiny to be used. Embryogenesis induction proficiency was genotype and explant specific (Nick *et al.,* 1986) whereby petioles of accession JN1 had 85% callus induction while JRV1, JCO4, JE4 and JNY1 had 67.5%, 66.25%, 65.0% and 17.5% respectively (Table 4.3) as observed in cotton using petiole and leaf disc. The developmental stage of explants was crucial in regeneration whereby young explants portrayed high cell differentiation. Induction of callus was the critical stage in this study where the type

and quality of callus influenced subsequent plant regenerations. Plant growth regulators, especially cytokinins and auxins alone or in combination play a very important role in callus induction process and its proliferation (Thomas *et al.,* 2006). Similarly, in this study it was observed that combination of BAP, KIN, IAA and TDZ is essential for high frequency induction of callus and multiple shoots. However, finding out the triggering combination of these plant growth regulators was the most important

(Timir *et al.,* 2007). Although BAP has been reported to be more effective than other cytokinins in micropropagation of various members of the Euphorbiaceae (Tideman *et al.,* 1982; Ripley *et al.,* 1986), TDZ was found to play an important role in callus induction in this study. When BAP, KIN and IAA were used on nodal explants (Kalimuthu *et al.,* 2007) they induced somatic embryogenesis without TDZ. The differential response of cytokinins and auxin in this case, is attributed to differences in uptake, levels of endogenous growth regulators and recognition by cells (Pedrose *et al.,* 1995; Souter *et al.,* 2000).

Low concentration of TDZ was maintained throughout the regeneration process and especially during shoot elongation as high concentrations have inhibitory effects (Preece *et al.,* 1991; Huetteman *et al.,* 1993; Feyissa *et al.,* 2005). Combination of 1.5 mg/l BAP with 0.6 mg/l KIN, 0.5 mg/l IAA and 0.1 mg/l TDZ promoted the highest callus induction of 85% within 8 weeks on all accessions in comparison to lower or higher concentrations. The rate of somatic embryo induction was accession specific (Shibli *et al.,* 2001). Within the JN1 accessions the highest rate of 85% was observed

and within JNY1 the lowest rate of 18% on was recorded on MS supplemented with 1.5 mg/l BAP with 0.6 mg/l KIN, 0.5 mg/l IAA and 0.1 mg/l TDZ. JCO4, JRV1 and JE4 accessions recorded 66%, 67.5% and 65% respectively. According to Ghimire *et al.,* (2010) leaf explants produced more callus than petiole explants. This agrees with the findings of this study where callus formation on leaf explants was significantly higher than petiole explants.

Majority of the calli differentiated to embryogenic calli while others to non embryogenic calli. Subculturing of embryogenic calli on SIM formed shoots at varied levels within 10-12 weeks. During embryo development heart, globular and torpedo shaped embryos were observed which originated from both compact and friable calli. The embryos germinated into shoots in all accessions except in JNY1 where cultures senesced without producing shoots. *In vitro* regeneration of *Jatropha curcas* is therefore genotype dependent (Kumar 2008; Kumar *et al.,* 2010 and 2010a).

Of the four concentrations tested 1.5 mg/l BAP with 0.6 mg/l KIN, 0.5 mg/l IAA and 0.1 mg/l TDZ gave the highest shoot length of 8.25cm in JNI accession while 0.5 mg/l BAP with 0.4 mg/l KIN, 0.3 mg/l IAA and 0.1 mg/l TDZ gave the lowest of 1.25cm in JCO4 accession. Efforts were made to further increase shoot multiplication and elongation by maintaining the plantlets on the same medium. In medium A and B low elongation was observed which according to Kumar *et al.,* 2008 is due to the profuse callusing at the basal end. The critical step of inducing roots on the elongated plantlets was obtained on the transfer of the 16 weeks old plants on rooting medium of ½ MS

supplemented with 3.0mg/l IBA and 3.5 mg/l NAA for 4 weeks. This resulted in a significant difference in the mean of average root number (3.50 JN1 accessions, 3.38 JCO4 accessions, 3.50 JE4 accessions and 3.50 JRV1 accessions) at P ≤0.05 (Shrivastava *et al.,* 2008).The acclimatization of the rooted shoots was accomplished successfully after transfer to pots containing forest soil, sand and manure in the ratio of 2: 1:1 under greenhouse conditions.

In all developmental stages the use of MS in the absence of plant growth regulators led to senesces of the explants used. This establishes well the role of cytokinins and auxins in different stages of somatic embryogenesis as reported by Fujimara *et al.,* (1980); Lo Schiavo *et al.,* 1989; Litz *et al.,* (1995). The most important research finding in his study was discovering the triggering combination and concentration of plant growth regulators besides other factors like type of tissue of a plant. It also confirms importance of low levels auxins somatic embryogenesis.

In this study the type of explants used in regeneration was an important factor (Shen *et al.,* 2008). Both the petiole and leaf disc explants showed differences in the percentage of embryogenic callus induction, probably due to differences in the levels of endogenous hormones, during the regeneration period (Preece *et al.,* 1991). The combination of 1.5 mg/L BAP with 0.6 mg/ L KIN, 0.5 mg/ L IAA and 0.1 mg/ L TDZ led to a successful regeneration. The plant survival (20%) in the greenhouse indicates a successful establishment of an efficient *In vitro* regeneration protocol for

Jatropha curcas. This research finding could be useful in producing true to type plants of Jatropha in mass and genetic transformation for desirable traits.

4.6 Conclusion

The regeneration protocol was significantly influenced by the type and concentration of PGR, explant type and genotype. Petioles and leaf discs of Jatropha were successfully surface sterilized using 70% ethanol for 1 minute and 20% Sodium hypochlorite with an exposure time of 20 minutes. Highest rate of embryogenic callus induction, embryo germination, shoot multiplication and elongation was achieved using 1.5 mg/l BAP, 0.6 mg/l KIN, 0.5 mg/l IAA and 0.1 mg/l TDZ. Root formation was achieved using 3.0 mg/l IBA and 3.5 mg/l NAA. The developed protocol gave reproducible results with CO4, JRV1, JN1 and JE4 genotypes but not with JNY1.

4.7 Recommendations

Optimization of the developed protocol is required for high production levels. Further investigation on greenhouse acclimatization is needed. The developed protocol can be used in genetic engineering of desired traits to come up with improved varieties.

CHAPTER FIVE

5.0 MORPHOLOGICAL CHARACTERIZATION AND YIELD POTENTIAL OF SELECTED KENYAN JATROPHA GERMPLASM

5.1 Abstract

Jatropha curcas is a potential species for biodiesel production in tropical and sub tropical regions of the world, varietal improvement and yield potential have not been documented in Kenyan germplasm. The objective of this study was to characterize selected Kenyan germplasm using morphological characters and assess their yield potential. Eleven Jatropha accessions collected from different regions in Kenya and later planted on an experimental plot at JKUAT farm were evaluated. A randomized complete block design was used. Data on stem diameter, plant height, branch number, branch length, leaf length and leaf number was collected after every two months.

The data were subjected to multivariate analysis using Principal Component Analysis (PCA) and clustering criteria. The results indicate that the characters contributing most variability are stem height, branch number, branch length and leaf length. Dendrogram generated through agglomerative hierarchical clustering revealed four main groups. Cluster A had Rift valley, Eastern and Coast accessions, B and C had Eastern and Rift valley accessions while cluster D had only one accession from Rift valley. Possibly cluster D accessions were introduced recently from Tanzania through Namanga boarder and has not spread to other regions in the country.

Morphological diversity estimated in the present study showed that there is variability in Kenyan Jatropha germplasm which can be used in conservation, genetic

improvement, breeding of higher yielding varieties or screen for cultivars resistant to pests and diseases. However further confirmatory studies using genetic characterization to accurately detect and classify the Jatropha accessions grown in Kenya are required.

5.2 Introduction

Jatropha curcas belonging to *Euphorbiaceae* family is perennial medium sized multipurpose tree (Openshaw, 2000; Kumar *et al.,* 2008). Morphologically, the stem is woody at the base and succulent at the top with spreading branches. It has large green to pale green deciduous leaves with several yellowish flowers and 2.5 to 4 cm long bell-shaped green capsules (fruits), which turn yellow when ripe (Little *et al.,* 1974; Morton, 1977).

Morphological markers are the oldest and are occasionally used for estimating genetic diversity but are not successful due to strong influence of the environment and are subject to individual bias (Tanksley, 1983). An understanding of the degree of genetic variation in populations of Jatropha is critical for improvement programs. Three varieties of Jatropha are occasionally mentioned: the Cape Verde variety which has spread all over the world, the Nicaraguan variety with few but larger fruits and a non-toxic Mexican variety which has traces of phorbol esters in the fruit (Heller, 1996; Henning, 1997; Sujatha *et al.,* 2005). This classification is based upon the size or the content of toxic molecules within the seed and it is not constant.

Some studies in Thailand have detected potential important variations although insignificant like in fruit and seed yield when plants are grown on a common site indicating low genetic variation (Sakaguchi *et al.,* 1987). Limited morphological variability has been reported in other records (Heller, 1996; Sukarin *et al.,* 1987). Correlating morphological characteristics like plant height, collar height and thickness, number of primary branches, petiole length, number of fruits per cluster, pedicel length and seed yield and oil content showed higher phenotype variations than genotype, indicating a predominant role of the environment (Sunil *et al.,* 2008 and Mishra, 2009).

Although Jatropha was introduced to Kenya, by the colonials in the 16th century mainly in the Kenyan coast and its Namanga boarder there is limited information with regard to the number of introductions, spread as well as the morphological variation of Jatropha populations grown in different parts of the country. The species is cultivated in Kitui, Thika, Namanga, Kajiado, Malindi, Nyanza, Nakuru, Marakwet, Naivasha and in Meru (Muok and Kallback, 2008). Tremendous significance of its potential as a biofuel crop has led to recent introductions especially from Asia. Since it is highly open pollinated and can produce seeds for a period of 50 years (Aker, 1997), a lot of changes have occurred resulting in loss of identity of all the introductions. It is possible that many new genotypes have arisen owing to many years of uncontrolled open pollination. This has led to difficulties in identifying Jatropha accessions from different regions of Kenya creating a desire for a better knowledge on its genetic diversity.

The objective of this study was therefore to access the morphological diversity in the Jatropha germplasm in Kenya and find out if there are any useful variations which can be used for improvement.

5.3 Materials and methods

5.3.1 Plant material

The accessions used in this study were collected from Jatropha growing regions (see section 3.3.1). Ninety six accessions were initially collected (Table 3.2). The plant material preparation involved two stages: i.) general assessment of seed germination and sprouting of cuttings and their growth rate in IBR green house ii) Transplanting and management in an experimental plot at IBR orchard in JKUAT. Sowing of seeds was done in trays and potting bags containing sand while planting of cuttings was on potting bags containing well mixed forest soil, sand and manure in the ration of 2:1:1. Seeds were sown in the soil at a depth of 3 cm (Henning, 2000b). About 2/3 of total length of cutting was in the ground and at least 2 nodes above the ground at a 45° angle. Rooting hormone was not applied on the cuttings in order to test whether the cuttings can sprout without hormone application. After the two stages only 11 accessions had 12 or more plants each which could be used for the field experiment while the rest had less due to low seed germination and low sprouting rate of cuttings leading to over presentation of some regions like Namanga. Nine of the accessions were raised from seeds and 2 accessions from cuttings. The plants were later transplanted in the experimental field (Table 5.1).

5.3.2 Field experiment: morphological

JKUAT is located in central province of Kenya at Latitude 1° 05´S, longitude 37° 00´ E and at an altitude of 1,525M above sea level. Rainfall (200M to 2000M) is bimodal with temperatures ranges from a minimum of 14°C (during cold season) to a maximum of 30°C (during dry season). The experiment was laid out in a randomized complete block design (RCBD) in a squared plot with three replicates each having four plants per accession at spacing of 2 m x 2 m within rows and 2 m x 2 m between rows. The depth and width of the holes was 1.5 feet x 1.5 feet. Farmyard manure of 2 kg per pit was applied at the time of planting. Irrigation of 2 litres per hole was regularly done (twice a week) in absence of rainfall. In the sixth month after planting the following traits were evaluated at an interval of two months: stem diameter, stem height, branch number, branch height, leaf length of the 3rd and 4th leaf on main stem and leaf number. Stem diameter (cm) was measured at the main stem base, stem height (cm) was measured from the base to the apex of the main stem, branch number was evaluated by counting the number of branches from the trunk, branch length was measured from the base to the apex of the branch, leaf length was measured from the petiole to the tip of the 4th and 5th leaf of the main stem, leaf number evaluated by counting the number of leaves in a plant.

Table 5.1: *Jatropha curcas* accessions used in germination and sprouting studies

Name	Code of accession	Plant material	County
Kitui G.A.F (KIGAF)	JE1	Cutting	Kitui
Kitui V.F (KIVF)	JE4	Cutting	Kitui

Tharaka 1(Tha1)	JE24	Seedling	Tharaka
Tharaka 4 (Tha 4)	JE26	Seedling	Tharaka
Kibwezi (Kibwe)	JE15	Seedling	Makueni
Markebuni (Mark)	JCO4	Seedling	Mombasa
Bita	JCO3	Seedling	Mombasa
Namanga 1 (Nam1)	JRV1	Seedling	Kajiado
Namanga 2 (Nam2)	JRV2	Seedling	Kajiado
Namanga 3 (Nam3)	JRV3	Seedling	Kajiado
Namanga 6 (Nam6)	JRV5	Seedling	Kajiado

5.3.3 Data analysis

Morphological data was submitted to Principal Components Analysis (PCA) using the XLSTAT 2008 statistical pacakge. Cluster analysis was carried out on the principal components with Eigenvalues of 1.128 to 3.147 using the Neighbor Joining method (Nei, 1973) or hierarchic ascendant analysis and Euclidian average distance.

5.4 Results

5.4.1 Germination and growth rate of Jatropha seeds

Out of 315 seeds planted a total number of 193 seeds germinated (62.15 %). Germination occurred in all trials with a significant difference in germination rate among accessions been identified. The highest germination rate among the trials was achieved on Namanga 2 accessions (72.3%) and lowest in Namanga 1 (50%). Trays and potting bag containers were used to study whether the type of container used affected time and rate of germination. The earliest germination was observed on

potting bags after 9 days while on trays after 11 days of sowing. All viable seeds germinated within 14 days (Table 5.2). The germination rate also varied from one accession to another whereby Namanga 2 accession had the highest germination rate of 72.3 % while Namanga 1 accessions had the lowest rate of 50 %. A significant difference in growth rate in terms of the plant height and leaf number was observed during the first eight weeks in the seven accession studied (Table 5.3).

Table 5.2: Mean germination of selected Jatropha accessions

Accession	Container type	Seed no.	% mean germination
Kibwezi	tray	45	62.3
Markebuni	tray	45	60.0
Tharaka 1	tray	45	54.3
Namanga 1	tray	45	50.0
Namanga 2	tray	45	72.3
Kibwezi	pot	45	57.6
Markebuni	pot	45	71.0
Tharaka 1	pot	45	61.0
Namanga 1	pot	45	70.0
Namanga 2	pot	45	63.0
Mean			62.15

Table 5.3: Mean growth rate of the selected Jatropha accessions at 8 weeks after transplanting

Accession	Mean leaf number	Mean height
Kibwezi	4.80	17.80
Markebuni	4.45	18.78
Tharaka 1	4.89	19.48
Namanga 1	4.46	22.60
Namanga 2	4.49	22.63

*Germination test was done on those accessions which had at least 100 seeds.

5.4.2 Sprouting rate of Jatropha cuttings

Two weeks after planting the cuttings started sprouting, different colours were observed on the shoot apex of Meru, Tharaka, Kibwezi and Magadi accessions, they were green, dark green, brown and maroon respectively (Plate 5.1 a, b. c and d).

During the plant growth and development all the four colours could be noted on different plants. A standard leaf lobe number of five was observed on all accessions both in the field survey, green house and in the experimental plot. A high sprouting rate of 60.0 % to 86. 7 % was recorded (Table 5. 4).

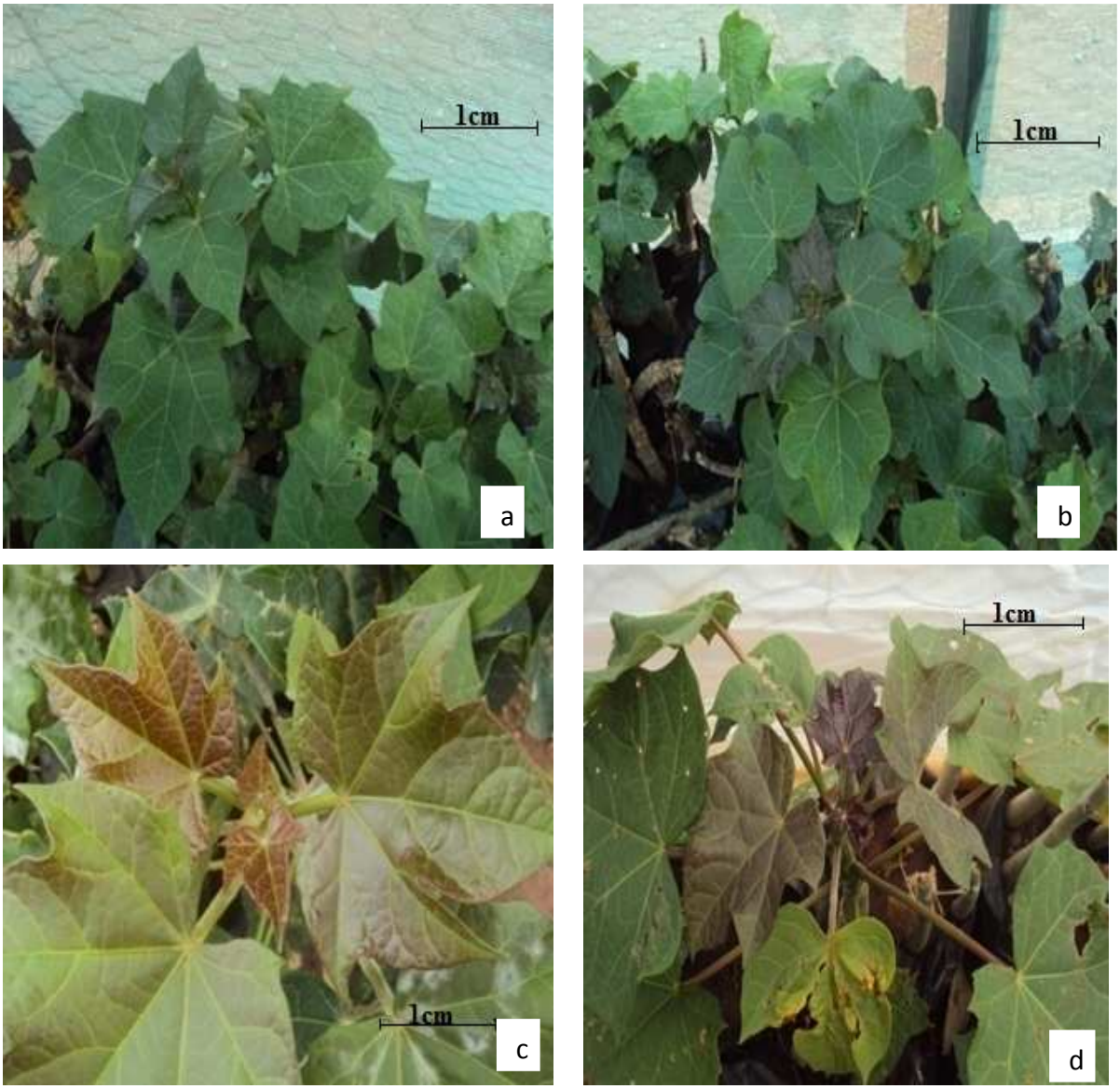


Plate 5.1: leaf colour of different Jatropha accessions a) Meru cuttings with green leaves, b) Tharaka cuttings with dark green leaves, c) Kibwezi seedlings with brown leaves, d) Magadi seedlings with maroon leaves

Table 5.4: Shooting efficacy of Jatropha cuttings

Accession	Cutting no.	Cutting colour	Mean length (cm) of sprouted cuttings
Kibwezi	15	brown	66.7
Tharaka	15	dark green	60.0
Namanga	15	green	73.3
Kitui G.A.F	15	green	86.7
Kitui V.F	15	green	80.0
Magadi	15	maroon	66.7
Meru	15	green	73.3
Mean			72.39

5.4.3 Growth and development of Jatropha accessions

During field survey the crop had varied branching types ranging from on the ground asymmetrical (Plate 5.2 a) to on ground symmetrical (Plate 5.2 b) and above ground (Plate 5.2 c). Shedding of leaves during dry season was observed even in irrigated fields (Plate 5.2 b). Rooting morphology of seeds and cuttings generated plants was also studied where seeds generated plants had a strong taproot (Plate 5.3 a) while cuttings had fibrous rooting (Plate 5.3 b)

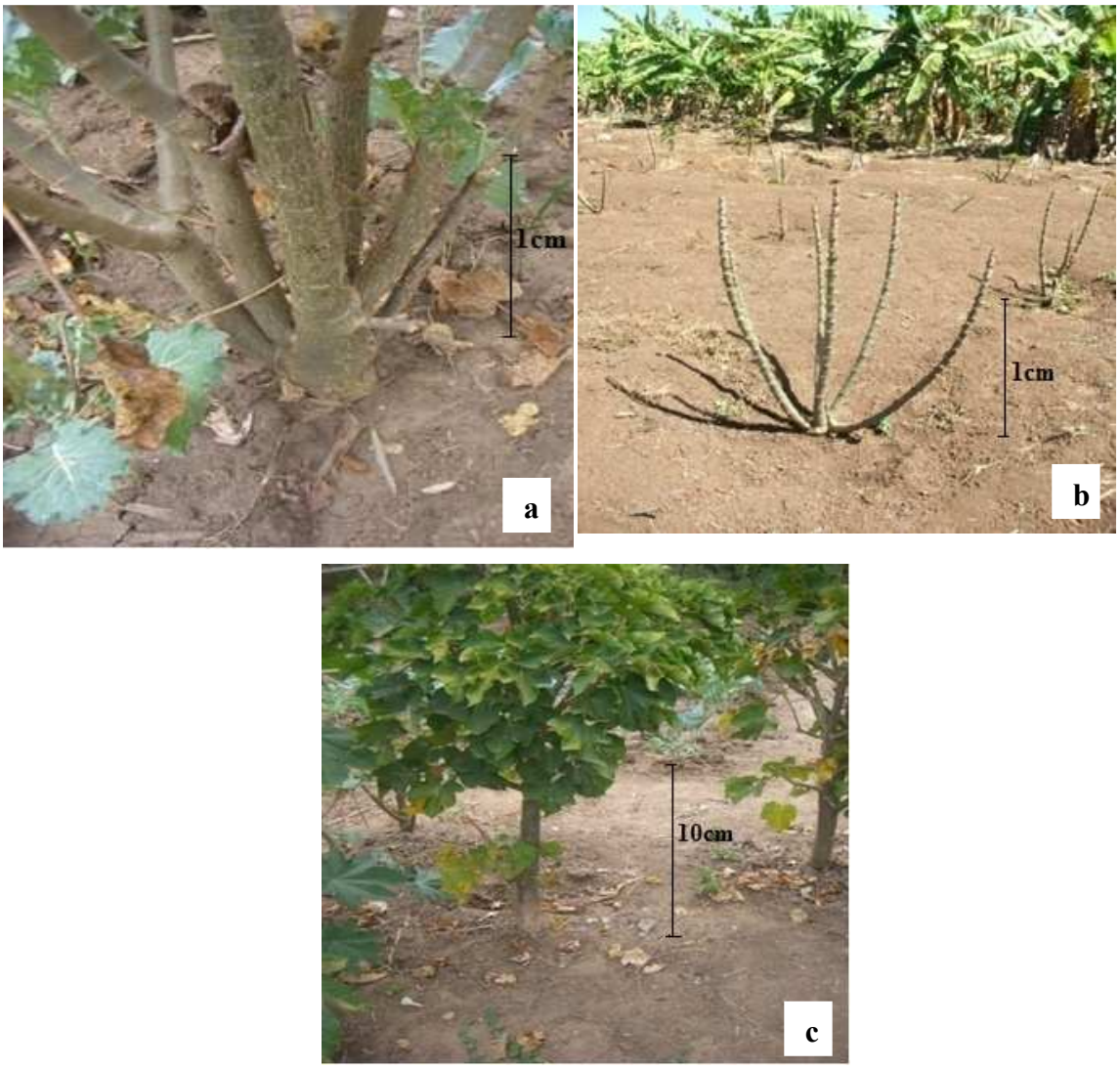


Plate 5.2: Jatropha branching a) on ground asymmetrical branching in Green Africa Foundation, Kitui b) on the ground symmetrical branching (IBR, JKUAT plot) c) above ground branching in Green Africa Foundation, Kitui.

Plate 5.3: Rooting morphology a) plants raised from seeds b) plants raised from cuttings

5.4.4 Morphological diversity in Kenyan Jatropha germplasm

Accessions tested displayed reasonable variation during early growth and development in all characters studied including stem height, branch number, branch height, leaf length, leaf number and stem diameter. Among the 11 accessions studied, mean stem height of between 22.99 and 55.17 cm classified the accessions into five groups, branch number of 1.58 to 3.78, branch length of 16.50 to 36.39c and leaf length of 8.16 to 14.31 cm gave three groups in each, leaf number of 12.58 to 22.38 two groups, stem diameter of between 7.88 and 10.83 cm produced one group (Table 5.5). These variations are an indication of some level of variability within the accessions. There was a clear indication of faster growth of plants propagated through cuttings compared to seeds. Kitui Vanilla Foundation and Kitui Green Africa Foundation generated

through cuttings had the highest measurements across all traits compared to the rest of planting material raised through seeds.

Table 5.5: Means of the different plant traits after 6 months of growth

Accession	Stem height	Branch number	Branch length	Leaf number	Leaf length	Stem diameter
KIGAF	52.89±4. 0[b]	3.78±0.21 [a]	35.35±4.2[ab]	19.86±1.16[ab]	14.01±0.64 [ab]	9.54±0.26[a]
KIVF	55.17±8.25[a]	3.75±0.17[a]	36.39±6.69 [a]	22.37±2.55a	13.59±1.23 [ab]	10.83±0.79[a]
Bita	42.65±3.19[abc]	2.64±0.49[ab]	35.92±3.96 [a]	19.11±1.83 [ab]	11.87±0.52 [ab]	9.21±0.53[a]
Mark	38.76±4.10 [abc]	2.11±0.20 [ab]	22.01±4.50 [ab]	19.04±2.3[ab]	17.22±2.44[b]	9.80±0.58[a]
Nam1	36.66±3.13 [abc]	3.46±0.79 [ab]	29.21±2.66 [ab]	18.15±1.33[ab]	13.27±0.50 [ab]	8.81±0.41 [a]
Tha1	36.52±3.19 [abc]	2.79±0.57 [ab]	26.24±2.43 [ab]	20.25±1.61 [ab]	13.80±1.60 [ab]	9.60±0.70[a]
Nam3	35.49±4.03[abc]	2.89±0.57 [ab]	29.68±3.86 [ab]	18.10±1.54 [ab]	14.31±0.96 [ab]	8.92±0.47[a]
Kibwe	35.46±5.30 [abc]	2.14±0.27 [ab]	23.96±2.94 [ab]	17.62±2.25 [ab]	12.55±1.28[ab]	8.50±0.96[a]
Tha4	31.46±3.59[bc]	2.67±0.38 [ab]	20.64±2.78 [ab]	18.11±143[ab]	12.16±0.95 [ab]	9.38±0.40[a]
Nam2	27.94±5.29[c]	1.63±0.17[b]	22.63±2.39[ab]	15.67±1.50 [ab]	10.91±1.12 [a]	7.88±0.97[a]
Nam6	22.99±6.22[c]	1.58±0.25[b]	16.50±3.99[b]	12.58±2.48[ab]	8.16±2.11[a]	8.67±1.57 [a]

Means (± SE) followed by different alphabets in each column were significantly different at P ≤0.05 using Tukey's test

Stem diameter (cm) - measured at 10cm from the ground.

Stem height (cm) - measured from the base to the apex of the main stem.

Branch number - evaluated by counting the number of branches from the trunk.

Branch length (cm) - measured from the base to the apex of the branch.

Leaf length (cm) - from the petiole to the tip of the 4th and 5th leaf of the main stem.

Leaf number - evaluated by counting the number of leaves in a plant.

5.4.5 Principal Component Analysis

The six principal components (PC1 to PC6) took into account 100% total phenotypic variance High Eigen value in PC1 explained most variable of 73.594. PC2 was

13.114% expressing a low variability. As the Eigen value decreased the cumulative variability increased totaling to 100% at PC 6 (Table 5.6).

The first principal components with coefficient values greater than 1.0 explained 73.6% of the total variance present in the data set. The first principal component comprised of characters related to stem height, leaf number, branch number and branch length. The second principal component accounted for 13.114% of the total variation and was highly associated with leaf length. The third component which accounted for 7.35% of the variation was mainly correlated to characters related to stem diameter. The fourth component accounted for 3.473% of the variation and was related to branch number. The fifth component accounted for 1.648% of the variation and was related to leaf number. The sixth component accounted for 0.818% of the variation and was related to stem height. The relationship of these principal components and quantitative variables is given in Table 5.7. These can be explained by the Eigen vector whereby all the variables have a positive factor at PC1 while in PC2 the first 3 have a negative while the last 3 have a positive factor.

Table 5.6: Eigen values and the cumulative variability of the different principal components.

	PC1	**PC 2**	**PC 3**	**PC 4**	**PC 5**	**PC 6**
Eigen value	4.416	0.787	0.441	0.208	0.099	0.049
% variance	73.594	13.114	7.354	3.473	1.648	0.818
Cumulative %	73.594	86.707	94.062	97.535	99.182	100.000

Variates distribution based on the PC-1 and PC-2 showed the phenotypic variation among the accessions and how widely dispersed they were along both axes (Figure

5.1). The two components explained a cumulative variability of 86.57%. Based on variate distribution, Markebuni accessions were the most distantly related to the first group. In the second group, accessions of Kitui Vanilla Foundation and Kitui Green Africa Foundation showed less similarity to that group. In the third quarter Namanga 6 was the most distant accessions in the group. The fourth quarter Tharaka 4 was the least similar in the group.

Table 5.7: Correlation between variables and factors

Characters	PC**1**	PC**2**	PC**3**	PC**4**	PC**5**	PC**6**
Stem height	**0.454***	-0.167	0.019	-0.345	-0.506	**0.625**
Branch number	**0.419**	-0.303	-0.048	**0.852**	-0.049	0.048
Branch length	**0.402**	-0.500	-0.326	-0.359	0.024	-0.594
Leaf number	**0.453**	0.162	-0.022	-0.137	**0.813**	0.298
Leaf length	**0.320**	**0.739**	-0.494	0.073	-0.257	-0.189
Stem diameter	**0.387**	0.242	**0.804**	-0.045	-0.117	-0.360

***Values in bold indicate the most relevant characters (>0.3) that contributed most to the variation of the particular component.

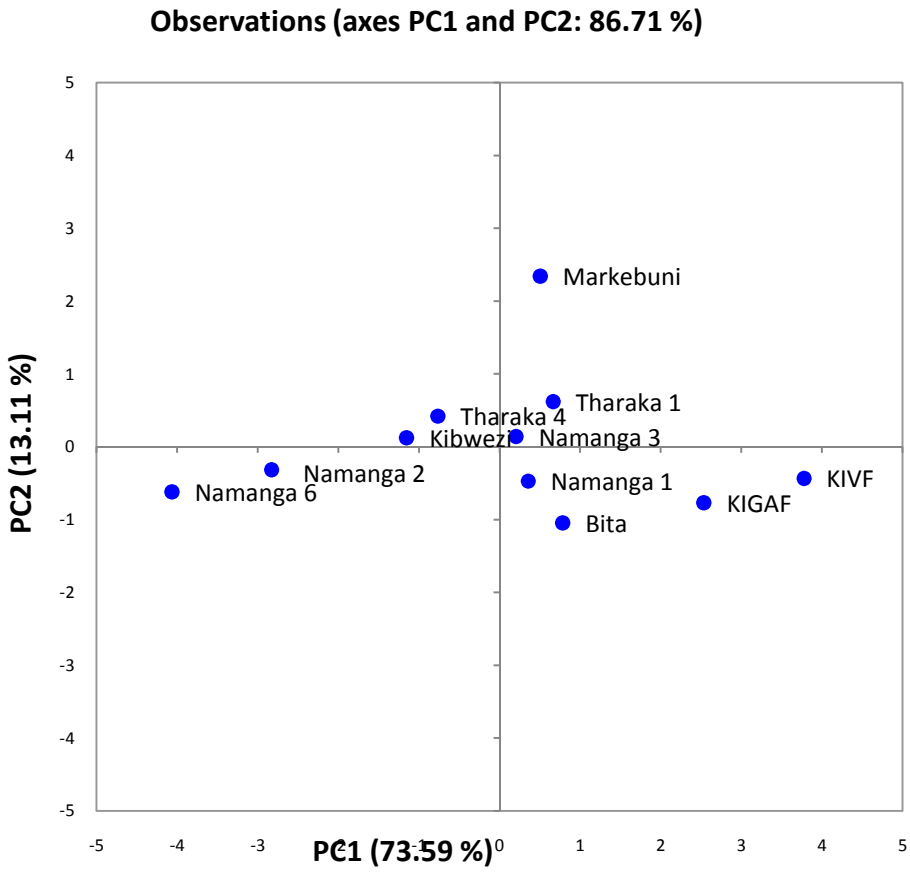


Figure 5.1: Distribution of variates in PC1 and PC2. PC1 accounts for 73.59% of the variation while PC2 accounts for 13.11 %.

The correlation analysis using Pearson (n) showed how the variables correlated in the PCA analysis in a pair wise manner. A number of variables were more correlated to each other than others. Stem height was highly correlated with branch length (0.874), leaf number (0.864), branch number (0.821) and stem diameter (0.784). Leaf length was moderately correlated with stem height (0.543) and stem diameter (0.518). Branch number was lowly correlated with leaf length (0.441). Branch length was noted to be less correlated with stem diameter (0.489) and lowly correlated with leaf length (0.348) (Table 5.6).

Table 5.8: Correlation matrix (Pearson (n)) for the variables calculated using PCA

Traits	Stem height	Branch number	Branch length	Leaf number	Leaf length	Stem diameter
Stem height	1	**0.821**	**0.874**	**0.864**	0.543	**0.748**
Branch number	**0.821**	1	**0.804**	**0.772**	0.441	**0.633**
Branch length	**0.874**	**0.804**	1	**0.746**	0.348	0.489
Leaf number	**0.864**	**0.772**	**0.746**	1	**0.714**	**0.783**
Leaf length	0.543	0.441	0.348	**0.714**	1	0.518
Stem diameter	**0.748**	**0.633**	0.489	**0.783**	0.518	1

Values in bold are significantly different from 1 with a significance level alpha=0.05

This is further elaborated in Figure 5.2 by the first and second Principal Component. The correlation among characters showed two main clusters of characters. The first cluster comprised of traits associated leaf and stem while second cluster comprised of traits associated with branch and stem.

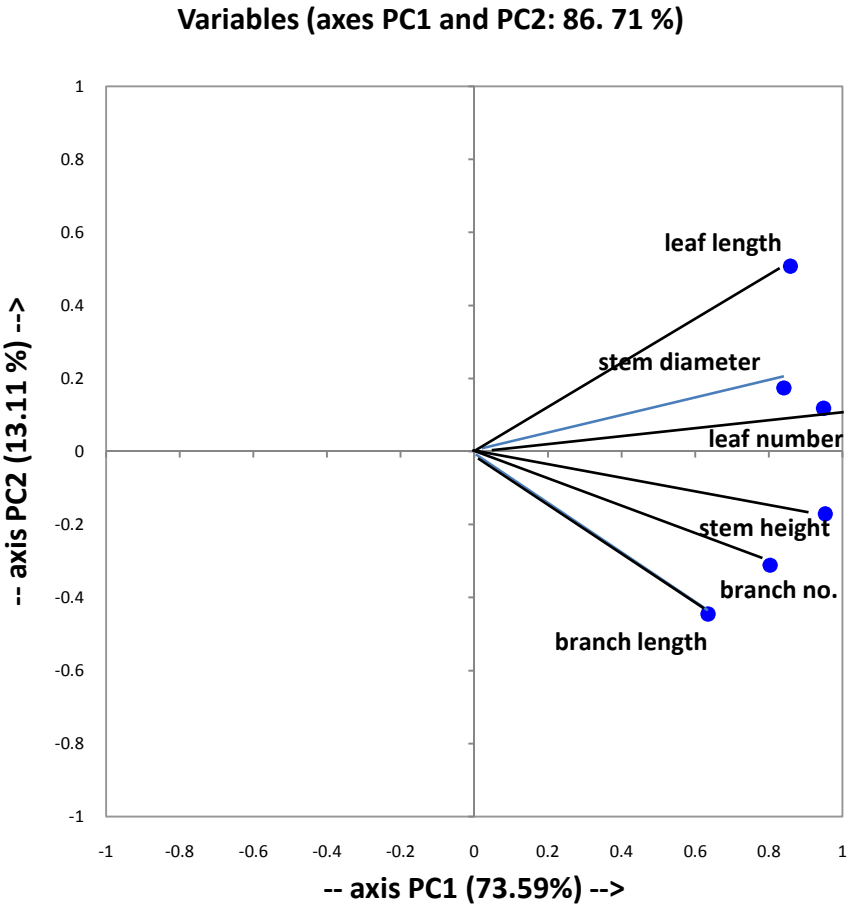


Figure 5.2: Correlation among characters associated with the first and second principal components. The closer the attributes are to each other in the PCA plot, the higher the correlation.

5.4.6 Cluster analysis

The agglomerative hierarchical clustering dendrogram illustrated the relationship among the accessions studied (Figure 5.3). At 1.0 level of dissimilarity, almost all 11 accessions were distinct from each other while at 7.5 level and above most accessions were similar to each other. The cluster analysis separated the 11 accessions from each other with Euclidean dissimilarity distance ranging from 1 to 25. The dendrogram at dissimilarity distance = 10 identified four main clusters; A, B, C and D based on the

major morphological characters used. Cluster A represented Tharaka 1, Tharaka 4, Markebuni, Namanga 10 and Bita accessions, cluster B had KIGAF , Namanga 3 and KIVF accessions, cluster C comprised of Kibwezi and Namanga 1 accessions and cluster D had Namanga 2 accession. Cluster A comprised of accessions from Rift valley, Eastern and Coastal regions while cluster B and C comprised of accessions from Rift valley and Eastern regions with cluster D having one accession from Rift valley.

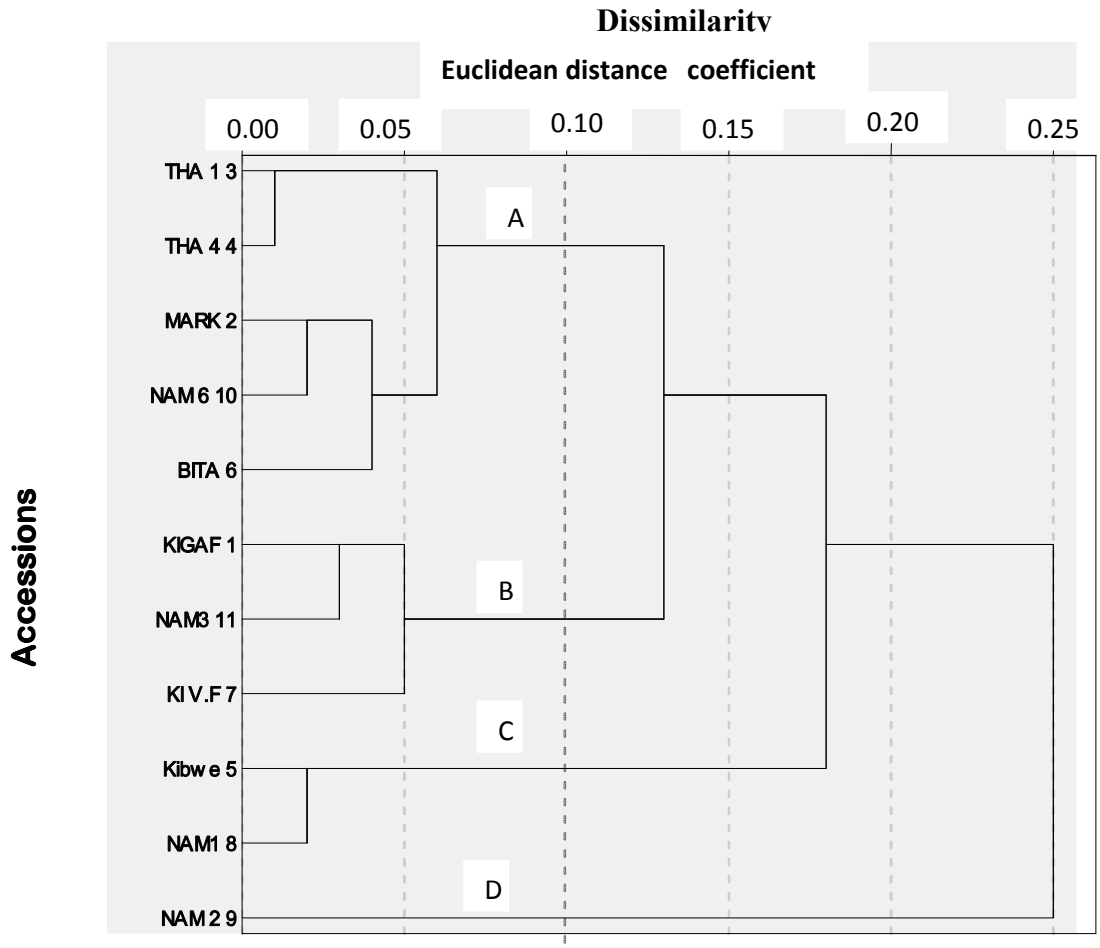


Figure 5.3: UPGMA Dendrogram (based on Euclidean distance coefficient) of 11 accessions generated by morphological characters.

5.5 Discussion

Morphological variability observed across Jatropha populations during a field survey was not consistent enough. This led to setting up an experimental plot with initial experiments on seed germination and sprouting of cuttings. Germination took 9 and 14 days in bags and trays, these results concur with those of Heller 1996; Henning 2000a who reported that it takes 10 days under good moisture conditions for Jatropha seeds to germinate. The results on seed germination efficacy of 62.15% were close to those

of Heller's 1992 of 68%. According to Kolbike, 1989 viability of Jatropha seeds decreases in respect to term of storage whereby seeds older than 15 months show viability below 50%. This is due to the oily nature of Jatropha seeds which does not allow for long storage. In our assessment studies, seeds were collected and stored in cool and well ventilated room conditions and sowed within 12 months hence no significant variation levels of viability was noted.

Storage term does not only reduce the germination rate but also oil content hence extraction of oil should be done few months after harvesting but not immediately to reduce oil content loss and impurities respectively. Other factors which reduce viability are seed quality, type of storage, pests and diseases. Cuttings had a high sprouting level of 72.39% compared to seed germination level of 62.06% although they lacked a taproot, hence not a good planting material for marginalized areas where good rooting system is required for moisture and nutrient scavenging. According to Heller, 1996 plants generated through cuttings portrayed a fast growth in the first year compared to seed generated plants. This agrees with our findings whereby the two accessions generated through cutting (Kitui Vanilla Foundation and Kitui Green Africa Foundation) performed better than others which were generated through cuttings.

Even though apical leaves of young cuttings during shooting in the greenhouse were observed to vary from one accession to another in colour this character was not constant in the field hence was not used as a phenotypic character. Most of the apical

leaves were mainly maroon. The level at which branching occurred was also limited with the same accession branching on the ground, above ground of both symmetrical and asymmetrical nature.

Morphological analysis based on six developed Jatropha morphological characters (section 5.3.2) showed levels of morphological polymorphism among the accessions of Jatropha in Kenyan, five accessions clustered together while other six clustered separately while one was isolated. This presence is an indication of diverse morphotypes at the individual genotype level showing possibilities of obtaining desirable trait combinations in specific accessions. Knowledge of correlations among characters is important in designing a breeding programme for any crop. In this study, strong correlations were identified between traits related to stem height and branch height, leaf number, branch number and stem diameter. This indicates possibilities of obtaining desirable trait by breeding accessions where these characters are highly expressed. Presence of trait variation among tested Jatropha accessions shows presence of possible opportunities for genetic improvement through direct selection of desirable accessions. This could be important in satisfying the rising demands by farmers, researchers and industries on this crop.

Levels of morphological variation among the various accessions may be due to recent introductions from Asia especially India (Isaac, 2008) where a lot of research has been done on the Jatropha germplasm. This could also have been as a result of farmers sourcing for planting material from different parts of the country other than sharing the

ones already existing within the neighborhood. Recent introductions from Tanzania through the Namanga boarder could have resulted to the isolation of Namanga 2 accession which seems to be a new variety found growing in Namanga. Cross pollination could also contribute to this diversity.

From the cluster analysis, the dendrogram showed that the first cluster (A) comprised of accessions from Tharaka, Markebuni, Namanga and Bita representing Eastern, Coast and Rift Valley regions showing possibility of spread of Coast and Namanga introductions to other parts of the country. The second and third clusters were composed of accessions from Eastern and Rift Valley regions a possibility of frequent planting material sharing between these two regions. The fourth cluster however had accessions from Rift valley meaning after introduction of this accession through the Namanga boarder, there was no planting material exchange with other regions or it's a new introduction from Tanzania by non governmental organization (NGO). It is clearly observed that Namanga accessions are spread in all the four groups which could mean that there was a wide spread of these accession to other parts of the country. This also concurs with this survey findings that the oldest Jatropha trees were mapped in Namanga of 50 years of age. This polymorphism could be environmental, propagation material or genotype dependant.

Although Jatropha is said to have fast growth (Sujatha *et al.,* 2005), resistant to pest and diseases, adapts well in marginal lands (Jones *et al.,* 1992), tolerates drought and therefore can grow in a semi arid regions (Henning , 1997), this was not the case in

this study. For quality data, watering twice a week was done during dry season, to improve soil fertility manure was added during planting, the plants were highly infested with pests and diseases hence a lot of pest and disease control was done using Redomil, Duduthrin and Dynamec as needed. These did not improve the growth of the plants that much whereby after 3 years the plants had not started flowering which limited my data collections.

5.6 Conclusion

Constraints of using only morphological traits for characterization of Jatropha germplasm were noted during this study. No single character was found to be sufficient to classify accessions phenotypically. The germplasm from Rift valley region presented the highest variation, being scattered all over the tree. This study has shown some variation in morphological traits of leaf, stem and branch among the different accessions of Jatropha. The analysis revealed some amount of diversity among 11 Jatropha accessions that can be used in selecting diverse parents in breeding programme. This is also crucial in utilizing the genetic potential of these genotypes for improvement of traits needed for adaptation to various conditions.

5.7 Recommendations

Further research involving characters related to the reproduction flowering and production like number of fruits per bunch, number of seeds per fruit and yield need to be done. However, there is the need to complement this work with other techniques such as using molecular markers to further accurately detect and classify the Jatropha accessions. This was done and is discussed in the next chapter.

CHAPTER SIX

6.0 MOLECULAR CHARACTERIZATION OF SELECTED KENYAN JATROPHA USING RAPD MARKERS

6.1 Abstract

Jatropha curcas (L.) is a species which is highly valued for its biodiesel potential as it produces non-edible oil seeds. In addition the species is a multipurpose plant thus making it the species of choice by small-scale farmers**.** However, despite the species attributes there is limited research on its genetic diversity and conservation in Kenya. This has restricted the species improvement hence limiting its prospects of being a successful candidate for biodiesel production. The present study was carried out to assess genetic diversity in a representative set of sixty nine accessions of Jatropha from Coast, Eastern, Rift valley, Western, Central and Nairobi regions of Kenya using random amplified polymorphic DNA (RAPD) markers. Four RAPD primers were used to generate 119 scorable polymorphic bands which were used to estimate genetic distances among populations and for construction of neighbour joining phenograms.

Analyses of Molecular Variance showed significant genetic difference with more variation (57 %; P = 0.01) among populations and less (43%; P = 0.01) variation within populations. No variation (0%; P > 0.01) across region was observed. Cluster analyses using UPGMA algorithm placed the 69 genotypes into 5 main clusters. The analyses separated the populations in accordance with their geographical origin where Central, Western, Coastal and Eastern were quite distinct while Rift valley and

Nairobi clustered together. This genetic diversity is important for genetic improvement of Jatropha.

6.2 Introduction

Jatropha also known as physic nut produces seeds containing 46-58% oil (Subramanian *et al.,* 2005). It is a biofuel crop which has aroused research interest on its genetic diversity with aim of improving it. Considering its cross pollination nature and vast semi wild distribution, it is expected to have a wide variation. In Kenya little work has been done so far on the germplasm collection and diversity evaluation in order to utilize and preserve this species. Understanding the genetic variability in plants is useful in breeding programme for improvement and genetic transformation for sustainable utilization. The key for success of any breeding programmes lies in a wide genetic variability and the availability of genotypes with desired traits (Heller, 1996). Priority should be to assess variability within and among the available germplasm and subject the material to improvement and multiplication.

Most varieties have resulted from selections made in the natural populations. The Cape Verde variety which has spread all over the world has medium seeds while the Nicaragua variety has fewer but larger fruits, yet their yield per ha seems to be the same (Heller, 1996; Henning, 2006). A nontoxic variety exists in Mexico, which is used for human consumption after roasting. This nontoxic accession does not contain phorbol esters and could be a potential source of oil for human consumption, the seed cake can be a good protein source for humans as well as livestock (Makkar, 1998).

The use of both molecular and morphological characterization is important in studying genetic variability. Use of morphological markers is often influenced by prevailing environmental conditions (Tanksley, 1983). Furthermore they are not suitable for perennial crops as it takes a long time to collect all the data. Molecular markers have proved to be valuable tools in the characterization and evaluation of genetic diversity within species (Nejia *et al.,* 2006). These markers are independent to the environmental influence, growth conditions, the type and age of tissue being analyzed (Vainstein *et al.,* 1994). Different markers have revealed different classes of variation (Powell *et al.,* 1996; Russel *et al.,* 1997). In Jatropha, markers have been successfully used in detecting genetic diversity and relationship (Ganesh *et al.,* 2008; Basha *et al.,* 2007). RAPD has several advantages, such as simplicity of use and a small amount of plant material is required (Nejia *et al.,* 2006). It is cheap and rapid method not requiring any information on the plant genome and has been used to study diversity of several plants (Belaj *et al.,* 2001; Deshwall *et al.,* 2005). It is locus specificity, has co - dominant nature, highly reproducible and gives substantial size of polymorphism (Wu *et al.,* 1999).

The objective of this study was to investigate DNA polymorphism in Jatropha, for analysis of genetic diversity between and among the Jatropha accession collected from various regions of Kenya.

6.3 Materials and methods

6.3.1 Accessions

Ninety six individual trees were randomly sampled from the six regions representing the species distribution range and areas of high germplasm (Section 4.3.1, Table 3.2). Within each region, young and healthy leaf tissues were collected randomly from 5 to 20 adult trees. A minimum distance of 100 m between individual trees was used to avoid the risk of selecting closely related individuals. The samples were immediately placed in well labeled polyethylene bags which were kept in cool boxes containing dry ice and then transported to IBR, JKUAT laboratory. The samples were stored at -70°C and later used for DNA extraction. Samples which gave poor DNA were further extracted five times. Sixty nine out of ninety six Jatropha accessions collected gave quality genomic DNA were used for RAPD analysis. Samples from the same region were grouped in the same population that is Eastern, Coast, Western, Rift valley, Nairobi and Central populations.

6.3.2 DNA extraction from Jatropha

The genomic DNA extraction in this study followed the CTAB (Cetyltrimethylammonium Bromide) DNA isolation protocol (Doyle and Doyle, 2002). Frozen leaf sample of 0.1g was weighed and ground into a pre-chilled mortar and pestle in liquid nitrogen to obtain a fine powder. The powder was transferred into 2.0 ml micro centrifuge tubes and preheated to 65ºC, 700µl fresh CTAB buffer having

2% Mercaptoethanol was added, the mixture was vortexed then incubated at 65ºC for 90 min with shaking after every15 min. The mixture was allowed to cool for 5 min, centrifuged at 13,000 rpm for 10 min at room temperature (RT). The supernatant was pipetted out into a fresh tube. An equal volume of chloroform: isoamyl alcohol mixture (24:1) was added to the extract and mixed by gentle inversion to form a uniform emulsion. The mixture was centrifuged at 13,000 rpm for 10 min at room temperature (RT). The supernatant was removed and Chloroform: isoamyl alcohol extraction step was repeated again. The aqueous phase was pipetted into fresh tubes gently, avoiding the interface. To the above solution, 5 M NaCl (to final concentration 2 M) and 0.6 volumes of isopropanol was added and incubated at RT for 1 hr, further DNA precipitation by addition of 500µl absolute ethanol followed by incubation for 30 min at room temperature was done. After incubation, the mixture was centrifuged at 13,000 rpm for 15 min. The white/translucent pellet was washed with 70% ethanol twice, dried and resuspended in 50 µl of TE buffer.

6.3.3 DNA purification

The sample was then treated with 20 µl of 10 mg/ml of RNase and incubated at 37 ºC for 1 hr. After incubation with RNase, one volume of Tris saturated phenol (pH 8.0) was added and mixed gently by inverting the micro centrifuge tube till it formed a milky white emulsion. The emulsion was centrifuged at 10,000 rpm for 5 min at RT. The supernatant was pipetted out into a fresh tube. The sample was then extracted with equal volumes of chloroform: isoamyl alcohol (24:1) twice. The DNA was

reprecipitated with 0.6 volumes of isopropanol, 2.0 M NaCl (final concentration) and incubated for 10 min. To the above, 20 µl of sodium acetate and 1 volume of 80% ethanol were added, incubated for 30 min and centrifuged at 10,000 rpm for 15 min to pellet the DNA. The pellet was then washed with 70% ethanol twice, air dried and finally suspended in 50 µl of TE buffer.

6.3.4 DNA quantity and quality determination

The yield of the extracted DNA was quantified using the agarose gel electrophoresis. Agarose powder (1.0 g) was dissolved in 100 ml of Tris Borate EDTA (TBE) buffer (1.0% w/v). The gel solution was stirred, brought to boil in a microwave for 2 minutes to completely dissolve the agarose then ethidium bromide at a concentration of 1mg/ml was added to the solution at 55^0C to facilitate visualization of DNA under ultra violet (UV) light. The gel solution was poured in a casting tray having a comb and left to gel (polymerise) for 30 minutes before removing the comb. It was then immersed in the electrophoresis tank containing 1 X TBE buffer. Each DNA sample (5µl) containing 2µl of loading dye was loaded in the wells. DNA ladder (100 bp) of 1µl equivalent to 25ng was used as a molecular weight marker and was run in parallel in one lane of the gel. The gel was run at a constant voltage of 80 volts until the bromo-phenol blue migrated almost to the end of the gel. The gel was then removed from the tank, placed in a UV trans-illuminator and photographed. The brightness of extracted DNA bands and ladder DNA were compared and quantity determined.

Quality of DNA was determined by measuring the absorbance of light (260 nm) in a spectrophotometer. To each sample of 2µl, 98µl TE was added, mixed well, and OD260 and OD280 read to determine purity. After UV quantification, the concentration of each DNA sample was adjusted to a concentration of choice with TE and stored at 4°C (Sambrook et al., 1989).

6.3.5 Polymerase Chain Reaction with RAPD primers

RAPD markers were used to reveal presence of significant differences among the germplasm based on the differential PCR amplification of DNA samples using short Oligonucleotide sequences. For accurate results, well to well and run to run consistency was optimized to minimise technical errors that might cause variability even within similar samples. This ensured that the amplification polymorphisms obtained are due to population variability and not cycler variability.

Four RAPD primers (Table 6.1) were selected from 10 primers as they gave better polymorphism compared to the others. Amplification was performed using PTC-100 thermal cycler (MJ research inc., USA). Roche Taq polymerase was used according to manufacturer's instructions. Amplification was carried out in a 25 µl mixture containing 0.5 µl of Taq, 2.0 µl (20-pmol) of primer, 2 µl of DNA, 3 µl of deoxyribonucleoside triphosphate (dNTPs) mix (2.5mM), 2.2µl $MgCl_2$, 5.0µl PCR 10x buffer, 2.0µl BSA and 8.3 µl of PCR water (Table 6.3). The control contained all the above except the DNA template. The reaction mixtures were subjected to the following temperature cycling profiles: Initial denaturation of the template at 94^{o}C for

3 minutes, 42 cycles were run with denaturation at 94^{o}C for 30 seconds, primer annealing at 27° - 30°C for 1 min (this depended on primer melting temperature) extension at 72^{o}C for 2.5 minutes and a final extension at 72^{o}C for 4 minutes. Ten RAPD primers were used to amplify the genomic DNA of Jatropha randomly and four primers which gave good polymorphic bands were selected.

Table 6.1: Hardwood RAPD Primers used in the study

Primer name	Sequence (5´- 3´), length	Tm(° C)	Anneal temp
KP1	GGC TCG TAC C 10 mer	34	28
KP2	CGT CCG TCA G 10 mer	34	28
KP3	GTT AGC GGC G 10 mer	34	28
KP4	CGG AGA GTA C 10 mer	32	27
KP5	CCT GGC GAG C 10 mer	36	30
KP6	TCC CGA CCT C 10 mer	34	28
KP7	CCA GGC GCA A 10 mer	34	28
KP8	AGC CGC TGG T 10 mer	34	28
KP9	GAC TGG AGC T 10 mer	32	27
KP10	ACG GTG CGC C 10 mer	36	30

Source: Kenya Forestry Research Institute, 2010.

The PCR conditions were optimized to come up with the best condition for all genotypes (Table 6.2). The PCR components which were optimized are Mg Cl_2, Taq Polymerase and water.

Table 6.2: PCR optimization conditions of the ten RAPD primers

	1st PCR trial	2nd PCR trial	Final optimization
PCR buffer with Mg Cl_2 (10X)	5.0µl	5.0µl	5.0 µl
dNTP(2.5 mM)	3.0µl	3.0µl	3.0 µl
Primer (20 pmol)	2.0µl	2.0µl	2.0 µl
Taq DNA Polymerase (5/µl)	0.2µl	0.4µl	0.5µl
PCR water	10.3µl	9.1µl	8.3 µl
Template DNA (25ng)	2.0µl	2.0µl	2.0 µl
Total volume	25.0µl	25.0µl	25.0 µl
	-ve results	- ve results	+ ve results

6.3.6 Agarose gel electrophoresis

After a successful amplification, PCR products were stored at 4 °C prior to electrophoresis. Agarose gel of 1.4% (w/v) was prepared as outlined in section 6.3.5. The PCR products (7ul) were mixed with 3ul of loading dye (Bromophenol blue) and loaded into the wells and 5ul 100 bp DNA ladder separate and subjected to electrophoresis at 80 V for 90 minutes (Sambrook *et al.,* 1989). The amplified products were visualized under ultraviolet light and photographed with gel documentation system (Gel LOGIC 200 imaging system – Kodak MI SE)

6.3.7 Data collection

Four RAPD primer results were used in the analysis. Each amplified RAPD fragment was visualized as a distinct band. For each primer, the presence or absence of bands in

each accession was visually scored and transformed into binary character matrix (1= presence, 0 = absence).

6.3.8 Data analysis

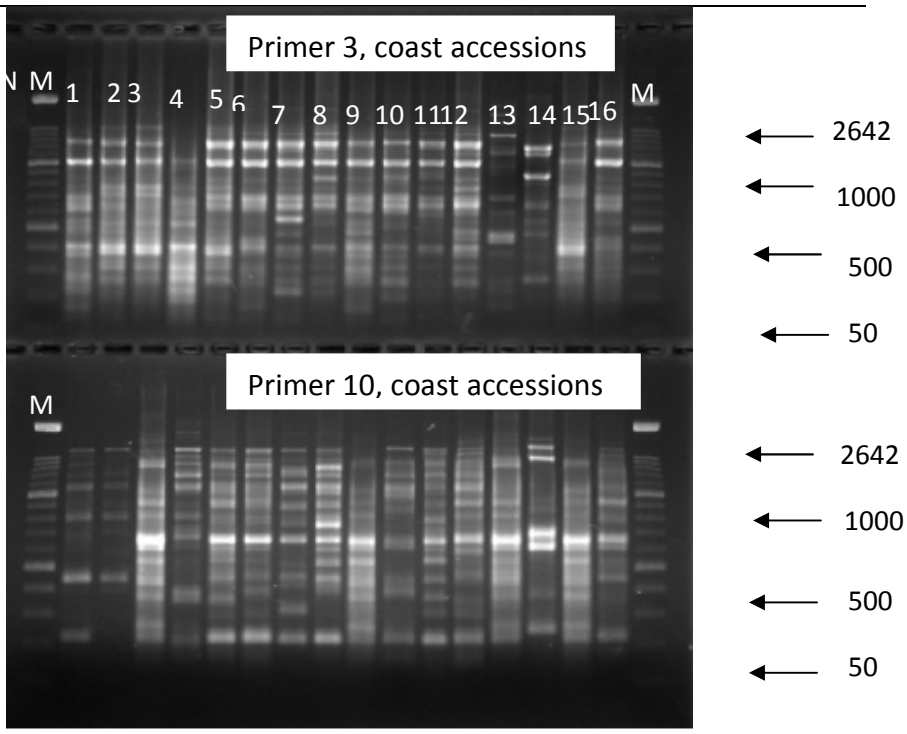
The percentage polymorphic loci were calculated for each population. The Nei's genetic distance matrix was used to generate the phylogenetic tree using un-weighted pair group arithmetic average (UPGMA) method in Molecular Evolutionary Genetics Analysis (MEGA) software (Tamura et al., 2007). Analysis of molecular variance (AMOVA) and principal coordinate analysis (PCA) were performed using GenAlEx 6.4 software (Peakall and Smouse, 2006).

6.4 Results

Out of 96 accessions collected, only 69 accessions gave quality and quantity DNA after extraction and were used in RAPD analysis. Four out of ten primers were screened to assess the genetic diversity among the 6 populations of Jatropha in Kenya. The four primers produced unambiguous polymorphic and reproducible fragments while the rest resulted in either no amplification or smeared profiles. These four primers were KFP 3, KFP 7, KFP 8 and KFP 10. They yielded 119 polymorphic loci ranging from 50 to 2642 bp in size. The number of amplified fragments per primer ranged from 24 to 30 with an average of 26 bands per primer (Table 6.3). Moderate diversity was observed from each primer with all showing over 55 % polymorphism. An example of the molecular profiles generated using KFP 3, KFP 10, KFP 7 and KFP 8 primers on Coast accessions is shown in Figure 6.1.

Table 6.3 : Primers polymorphism

Primer	No. of amplified bands	No. of polymorphic bands	Polymorphic bands %
KP3	24	18	75
KP7	30	19	63.3
KP8	26	15	57.7
KP10	25	14	56



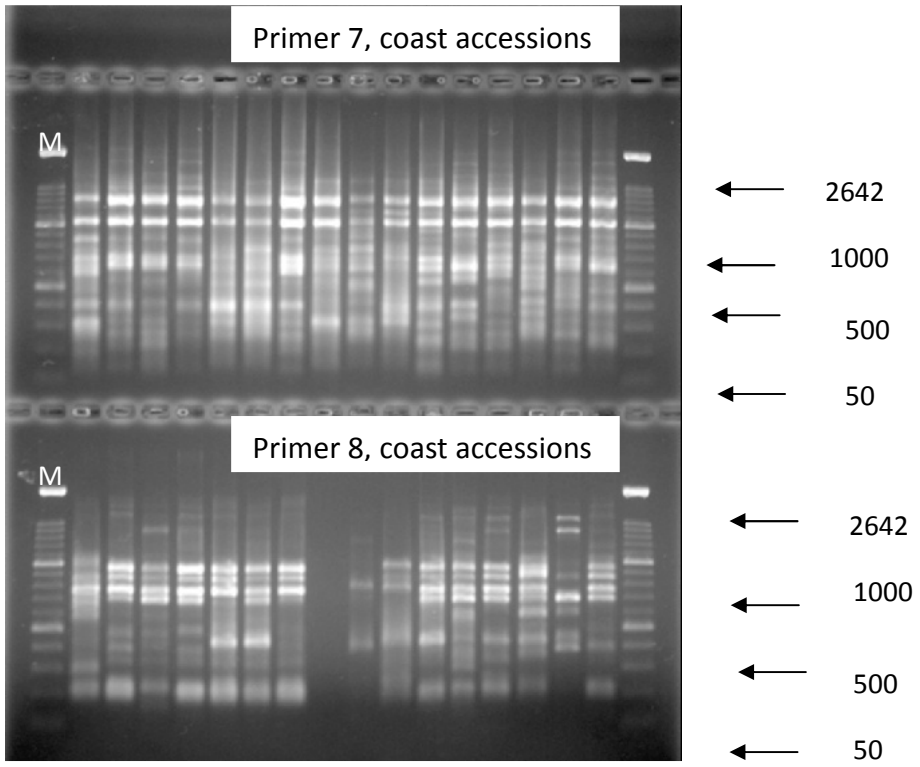


Figure 6.1: RAPD profiles of *J. curcas* Coast (1-16) amplified using primer KFP-3, KFP-10, KFP-7 and KFP-8. M is molecular weight marker (100 bp DNA ladder) by Roche.

To assess the overall distribution of diversity within and among these populations, an analysis of molecular variance (AMOVA) was done. AMOVA revealed significant variation among the populations (57 %) higher than within populations (43%). The overall genetic relationship among the 6 populations was summarized using both PCA and UPGMA cluster analysis. Results from both showed similar structuring obeying the underlying geographic regions (Figure 6.2 and 6.3).

Principal coordinate analysis divided the 69 genotypes into five groups. The first axis accounted for 33.66 % of the total variation while the second axis accounted for 21.29 % and clearly isolated Eastern population from others. The third axis accounted for

15.72 % and clearly isolated Coast population from others and the fourth 29.32 % which clearly isolated Central and Western populations from the others (Figure 6.2). Populations from Nairobi and Rift valley clamped together in axes one and three.

Jaccard's similarity coefficient of UPGMA cluster analysis was used to construct a dendrogram. It illustrated the overall genetic relationship among the 69 accessions of Jatropha studied. The dendrogram resulted from MEGA 4 program as shown in figure 6.3. Based on the dendrogram the 69 genotypes were grouped into 3 main clusters. Among the three clusters, cluster III was the largest comprising of 3 sub clusters. The first sub cluster included 24 accessions from Eastern region. The second sub cluster included 7 accessions from Coast, while the third sub cluster included 8 accessions from Nairobi and 1 accession from Coast. Cluster II was the second largest comprising of 21 accessions which further subdivided into two sub clusters. The first sub cluster included 16 accessions from Rift valley while the second sub cluster included 5 accessions from Western. Cluster I was the smallest comprising of 8 accessions from the Central region (Figure 6.3).

Table 6.4: Analysis of molecular variance of *Jatropha curcas* from 6 regions of Kenya

Source of variation	Degrees of freedom	Sum of Squares	Estimated Variance	Variation (%)	Differentiation Indexes	P-value
Among Regions	2	220.718	0.000	0%	PhiRT=0.038	p‹0.990
Among Pops	3	404.772	11.359	57%	PhiPR=0.568	p‹0.010
Within Pops	63	543.742	8.631	43%	PhiPT= 0.552	p‹0.010
Total	68	1169.232	19.990	100%		**0**

PhiRT: measure of genetic differentiation among regions for the total populations; *PhiPR:* measure of genetic differentiation among populations within a region; *PhiPT*: measure of genetic differentiation among populations.

Phi statistics indicated the presence of structuring and possibly genetic barriers. *PhiPT* differentiation index had a value of 0.552, which was significant, indicating moderate differentiation (43.0 % of total genetic variation due to differentiation within populations). The significant value of *PhiRT* showed that 3.8 % of total genetic variation was due to differentiation among regions.

The Principal coordinate analysis (PCA) of Jatropha individuals from Central, Western, Coast, Nairobi, Rift valley and Eastern populations based on Nei's epigenetic distance.

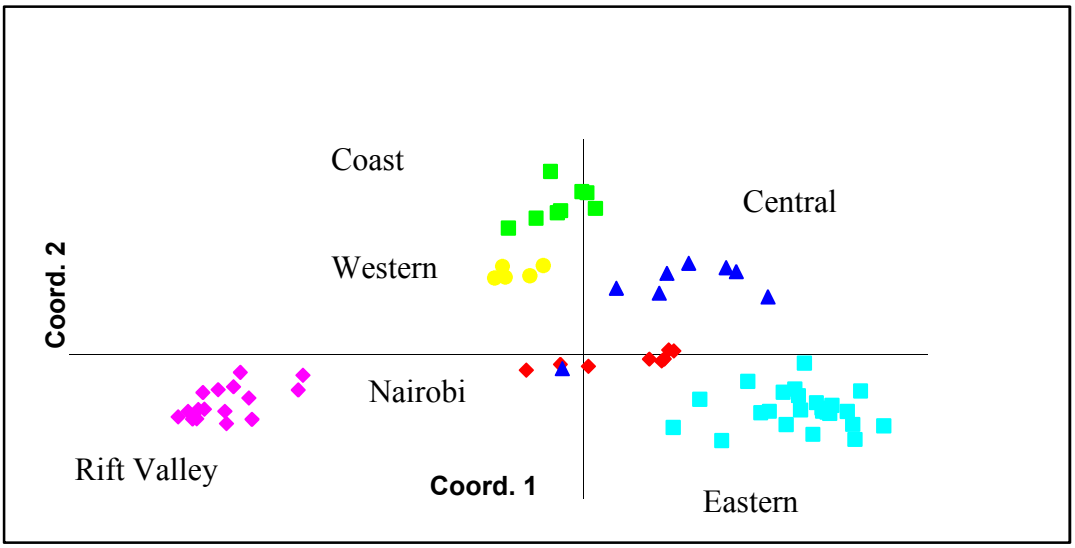


Figure 6.2: Principal coordinate analysis (PCA) of the 69 individuals of *J curcas* from 6 populations based on Nei's epigenetic distance.

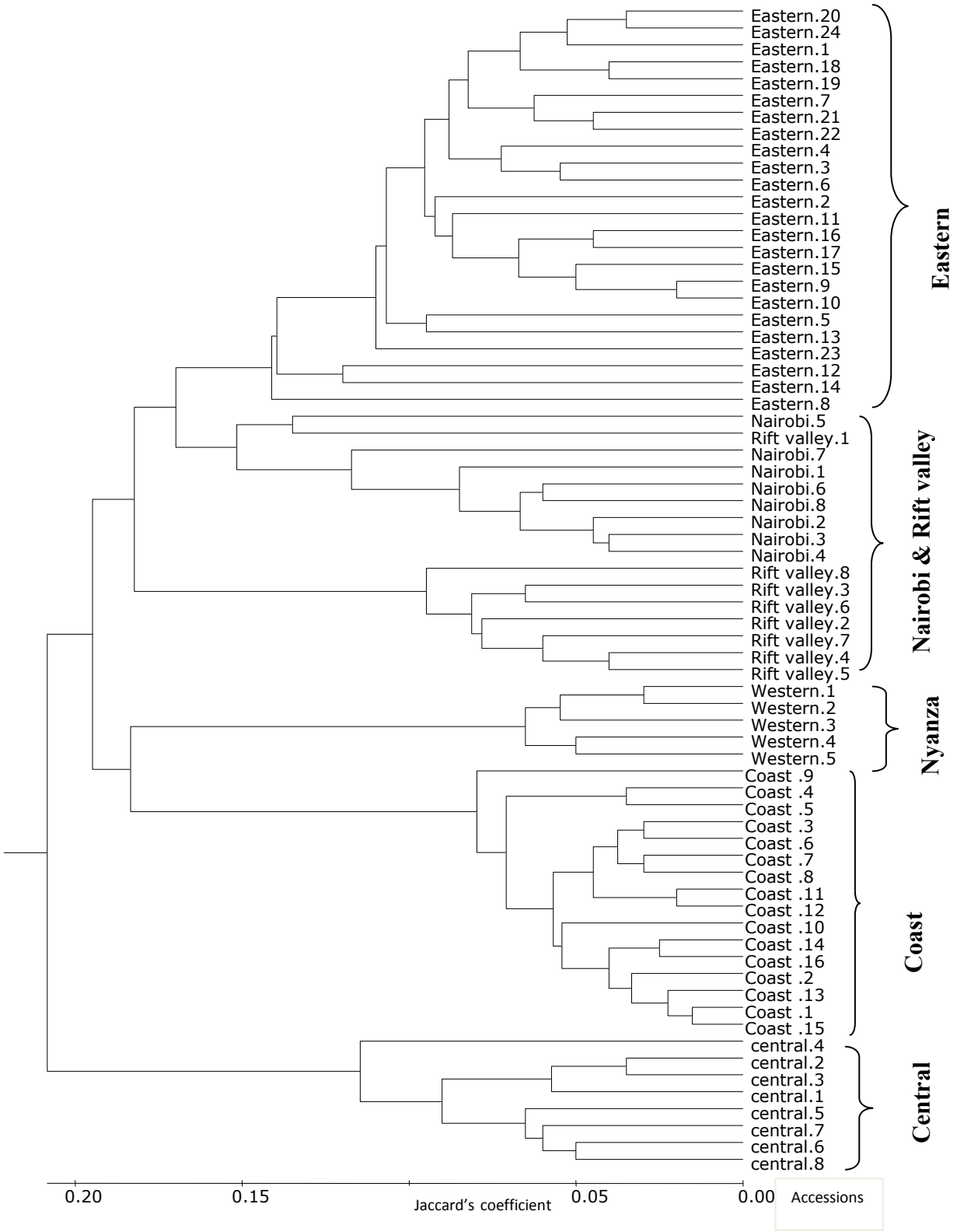


Figure 6.3: UPGMA dendrogram (based on Jaccard's coefficient) of 69 genotypes *Jatropha curcas* generated using RAPD markers.

6.5 Discussions

In this study 69 Jatropha genotypes from Kenya showed a moderate percentage of 57% polymorphism detected with 4 primers as compared to other studies in India by Ganesh *et al.,* (2008) and Subramanyam *et al.,* (2009) whom reported much higher polymorphism of 80% across 8 genotype and 75.2 % across 40 genotypes respectively indicating a wide genetic base. However Basha *et al.,* (2007) reported low levels of molecular diversity of 40% among India genotypes indicting a narrow genetic base. High genetic diversity among populations could be due to seed movement and gene flow (Padmesh *et al.,* 1999).

Variation within population was lower (43%) compared to among population (57 %). Generally there is low genetic diversity in this species as revealed by most studies which remain unclear (Singh *et al.,* 2010). According to Bhattacharya *et al.,* (2005), Jatropha is well adapted and has high stress tolerance that environmental influences can lead to low genetic make up change over long period of time. The fact that it is also a newly cultivated crop and not much improvement has been done which could also bring more genetic diversity. Low diversity within populations could be as a result of its vegetative means of propagation. In the rural areas, farmers also use cuttings instead of seeds which lead to narrow gene pool. Long distance seed dispersal is also limited, because the seeds are toxic hence animals do not feed on them which leads to blocked gene flow among populations (Xiang *et al.,* 2007; Ou *et al.,* 2009).

In general, studies have shown low genetic variation in *Jatropha curcas* from different populations using both morphological and molecular methods. Possibly it is due to the fact that most of these studies were done with introduction materials from Asia, South America and Africa while Central America which is the probable centre of origin remains less explored. Low genetic variation reported is a limiting factor towards genetic improvement. These findings calls for research on genetic diversity to be done on other regions like Naivasha, Nakuru, Marsabit and Marakwet as reported by Muok *et al.,* 2008.

Cluster analysis was able to determine both the degree of diversity and how the variation is distributed among regions, among populations and within populations. From the dendrogram constructed from the pooled data, this study grouped the genotypes as per geographical distribution. The 69 genotypes which were from different geographical regions were divided into three major clusters. Accessions from Central, Coast, Western and Eastern were isolated from each other. These might be as a result of more self pollination system of Jatropha leading to low gene flow between regions. Recent introductions by NGO's might not have affected the genetic diversity in these regions too. Rift valley and Nairobi population showed a common pool which is as a result of introductions made from Magadi recently as earlier shown in our study (Table 3.2). According to Muok *et al.,* (2008) Jatropha was introduced to Kenya by the Portuguese in the 16th century and probably in the coastal region then spread to other parts of the country. Some of the old introductions approximately 50 years old

were recorded in Rift valley (Namanga) and Eastern (Meru) regions during my field survey.

6.6 Conclusion

While there was limited genetic variation of 11 accession (Figure 5.3) of Kenyan Jatropha germplasm, DNA finger printing of 69 Kenyan Jatropha accessions has indicated a considerable genetic variation (Figure 6.3). The genetic relationships of the 69 Jatropha accessions are much closer to each other in the cluster analysis. The results of genetic diversity study provide estimates on the level of genetic variation among diverse materials that can be used in germplasm management, varietal protection and Jatropha improvement. The amount of genetic diversity found with RAPD markers is able to distinguish between breeding lines for varietal protection. The estimates of genetic similarity are particularly useful in choosing widely divergent parents with desirable traits for genetic mapping and selection.

6.7 Recommendation

Studies on further genetic diversity using other molecular markers and covering a wider range should be carried out. Studies on improvement through breeding and genetic engineering should be done using the available information whereby Jatropha transformability tests were done and are discussed in the next chapter.

CHAPTER SEVEN

7.0 EVALUATING GENETIC TRANSFORMABILITY OF KENYAN JATROPHA GERMPLASM

7.1 Abstract

Development of an efficient transformation protocol for Jatropha is important for genetic improvement of the potential biofuel feed stock plant. In this study transformation of 3 Jatropha accessions JCO4, JE4 and JN1 was investigated. The *Agrobacterium tumefaciens* strain EHA101 harboring the binary vector pTF102 was used to introduce GUS (ß-glucuronidase) gene into Jatropha leaf discs and petioles. Both leaf discs and petiole explants were cultured on MS medium supplemented with 1.5 mg/l BAP, 0.6 mg/l KIN, 0.5 mg/l IAA and 0.1 mg/l TDZ. Five stages; infection, co- cultivation, callus induction, embryo germination and selection in three replications involving complete randomized design were assessed. Data on callus, shoot formation and GUS expression was recorded during regeneration.

Data was analysed using Excel. Infected leaf discs exhibited more callus induction compared to petioles. This rate of callus induction greatly determined embryo and shoot formation. Four weeks after selection period, the evidence of GUS activity in transformed Jatropha shoot-tissues was histochemically confirmed. The percentage of GUS-positive shoots per total numbers of evaluated shoots was calculated to estimate the efficiency of transformation (44.43%). Results indicated that the percentage of GUS positive shoots varied among Jatropha accessions. The highest percentage of shoots with GUS activity (60.0 %) was observed in JE4 accessions and the lowest percentage (25.0 %) in JN1 accessions. High frequencies of GUS expression were

observed mostly in leaf discs of Jatropha shoots and less detected in petiole parts. Thus, it is possible to transform Jatropha and this offers the foundation for genetic improvement of Jatropha through genetic engineering approaches.

7.2 Introduction

Jatropha is a native to the South-American tropics and is distributed through out the tropics and sub-tropic marginal lands of the world. It is rated a high potential biodiesel crop world wide. Its seeds have a high oil content which can easily be converted to bio-diesel. The oil is also used in detergent, soap, cosmetics, drugs, biopesticide and fertilizer industries (Mazumdar *et al*., 2010). The plant is also used in reclaiming land, demarcations and in fencing homestead (Kaushik *et al.,* 2007).

However, full utilization of the plant oil is limited by low oil-bearing seed yield. Currently seed yields depend on various factors, such as environment (Openshaw, 2000), branching architecture, genetics and management (Gour, 2006). Limited branching is considered as one of the major factors limiting seed yield in Jatropha. Increasing the number of seed bearing branches can improve it (Gour, 2006; Abdelgadir *et al*., 2007). Although some traditional practices like manual pruning (Gour, 2006) and application of plant growth regulators under field conditions (Lovat *et al.,* 2006; Abdelgadir *et al*., 2007) are available for promoting Jatropha branching and healthy inflorescences, stability and efficiency of these still remain a major concern.

Recent studies have shown the possibility of optimizing the extent of branching by genetic modification in several plant species including Petunia (Bennett *et al.,* 2006), tobacco (Bereterbide *et al*., 2001) and *Arabidopsis* (McSteen *et al.,* 2005). Over expression of a zinc finger protein of petunia, designated Lateral shoot-Inducing Factor (*LIF*), in transgenic petunia, tobacco and *Arabidopsis* was found to dramatic increase lateral shoots and reduce the plants height. The plant responses to *LIF* over expression seem to be widely conserved at least among dicots hence *LIF* could serve as a tool for genetic manipulation of branching patterns of plants (Nakagawa *et al*., 2005). For Jatropha curcas, an efficient and stable transformation protocol need to be developed for successful introduction of desirable genes. Genetic enhancement and domestication of Jatropha should follow the same course as that of castor since the wild castor once considered as a minor oilseed crop has become a major industrial crop. Apart from increasing branching other traits like earlier maturity, non toxicity, resistance to pests and diseases, drought tolerance, higher ratio of female to male flowers and improved fuel properties can be studied (Sujatha, 2008).

Several reports on transformation in Jatropha have been documented. Li *et al*., (2008a), obtained *uidA* gene expression from regenerated plants arising from Jatropha cotyledons after *Agrobacterium* mediated transformation, with a low transformation frequency of 13%. Zong *et al.,* (2010) obtained 23.91 % lateral shoot-inducing factor gene expression using young leaves after *Agrobacterium* mediated transformation. Effects of age and orientation of the cotyledon explants on *Agrobacterium*-mediated transformation of Jatropha were studied by Mazumdar *et al*. (2010). Micro projectile

bombardment of embryo axes was reported by Mukul *et al.,* (2011) where 44.7 % transformation efficiency was reported. Here we report the results of the development of an *Agrobacterium*-mediated system for transformation of Jatropha through somatic embryogenesis using leaf disc and petiole on Kanamycin and Spectinomycin selection. Expression of *aad*A transgene and its evaluation by histochemical staining for GUS on transformants is also reported.

This study was carried out to test the transformability of the *in vitro* regeneration protocol developed in section 4.

7.3 Material and Methods

7.3.1 Experimental site and design

The laboratory experiments were conducted between August 2011 to January 2012 at Kenyatta University Plant transformation biosafety level 2 laboratory. The experiments were set up in completely randomized design (CRD). For experiments on sterilization, callus induction and shooting consisted of 3 replicates and were repeated 3 times.

7.3.2 Construct and *Agrobacterium tumefaciens* strain

Agrobacterium tumefaciens (*A. tumefaciens)* mediated transformation system of gene transfer was used in this study. EHA101 bacterial strain harboring the binary vector pTF102 (12.1kb) was used (Hood *et al.,* 1986). The 5.9-kb T-DNA region of this construct is shown in Figure 7.1. The vector is a derivative of the pPZP binary vector

(Hajdukiewicz *et al.,* 1994) that contains the right and left T-DNA border fragments from a nopaline strain of *A. tumefaciens*, a broad host origin of replication (pVS1) and a spectinomycin-resistant marker gene (*aad*A) for bacterial selection. The CaMV 35S promoter (P35S) was used to drive both the *bar* selectable marker gene and the *gus* reporter gene. A tobacco virus translational enhancer (Carrington and Freed, 1990) was included in the 5' end of the *bar* gene. The soybean vegetative storage protein terminator (Mason *et al.,* 1993) was cloned to the 3'end of the *bar* gene. The GUS gene contained a portable intron in its codon region (Vancanneyt *et al.,* 1990) to prevent GUS activity in *A. tumefaciens* cells. This vector system, pTF102 in EHA101, was maintained on yeast extract peptone (YEP) medium (An *et al.,* 1988) containing 100 mg/l spectinomycin (for pTF102) and 100 mg/l kanamycin (for EHA101). Bacteria cultures for weekly experiments were initiated from stock plates that were stored for up to 1 month at 4°C after being refreshed from long-term, - 80°C glycerol stocks. Bacteria cells were grown overnight at 28°C on a rotary shaker at 200 rpm. An aliquot (0.5 ml) of overnight culture was inoculated into 50 ml of liquid YEP medium containing the same antibiotics and allowed to grow at 28°C with vigorous shaking until the OD_{600} reached about 0.8. *A. tumefaciens* cells were then collected by centrifugation at 4000 rpm for 5 min at room temperature and resuspended in 100 ml of liquid MS medium for infections.

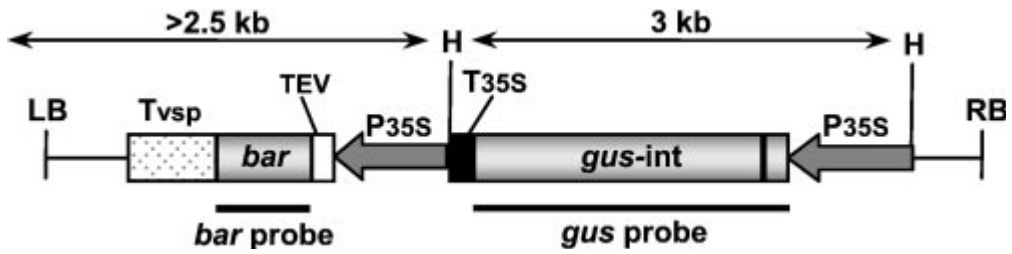


Figure 7. 1: T-DNA region of standard binary vector pTF102. LB, Left border; RB, right border; *bar*, phosphinothricin acetyltransferase gene; *gus*-int, _-glucuronidase gene containing an intron; P35S, CaMV 35S promoter; TEV, tobacco etch virus translational enhancer; Tvsp, soybean vegetative storage protein terminator; T35S, CaMV 35S terminator; H, *Hin*dIII (Plant Physiology, Vol. 129, 2002).

7.3.3 Plant material

Plant material was collected as shown in section 3, table 3.2. Seeds were used in this experiment. The embryos were removed from the interior seed coat and grown on hormone-free full-strength MS medium as in appendix 3 and cultured in a growth chamber at 25 ± 2°under a 16 hr light period and 8 hr dark period in air conditioned growth chambers illuminated by 40W (watts) provided by cool white fluorescent lights. The embryos were placed in glass beakers and kept in running tap water for 30 min to remove physical impurities. They were then transferred to new glass beakers containing 250ml of water into which 100µl/l Tween®20 (wetting agent) and 2ml/l of dettol detergent had been added. The beaker was swirled gently at intervals for fifteen minutes followed by rinsing (3 times) with distilled water to remove all the traces of detergent. Thereafter, they were kept in 0.3% (w/v) Redomil®(fungicide) plus 100µl/l Tween®20 for 1 hour then washed thoroughly with double distilled water. Under a clean Lamina flow chamber, the explants were subjected to 70% (v/v) ethanol for 30

sec, rinsed with double distilled water thrice to remove all the ethanol and then subjected to 20% (v/v) sodium hypochlorite containing 100µl/l Tween®20 for 20 min then rinsed three times with double distilled water. After sterilization, individual seed embryos were cultured on hormone free MS medium.

7.3.4 Media

Murashige and Skoog, medium supplemented with 1.5 mg/l BAP, 0.6 mg/l Kn, 0.5 mg/l IAA and 0.1 mg/l TD Z was used in the experiments. At each stage other components like antibiotics were added as required. Infection medium contained 68.4 g/l Sucrose and 36 g /l Glucose (pH 5.2) supplemented with 100µM Acetosyringone. Co cultivation media contained 30 g/l Sucrose, 100µM Acetosyringone, 2.8 g /l gelrite (pH 5.8) 500 mg/ l cefotaxime. Resting medium contained 30 g/l Sucrose, 250 mg/ l cefotaxime and 2.8 g /l gelrite (pH 5.8). Selection medium contained 30 g/l Sucrose, 100 mg/ l cefotaxime, 100 mg/ l Kanamycin, 100 mg/ l Spectinomycin and 2.8 g /l gelrite (pH 5.8). Infection medium was filter sterilized, whereas all other media were autoclaved and antibiotics were added later. MS media pH was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCL, solidified with 0.28 % (w/v) gelrite and autoclaved at 121°C for 20 min. Antibiotics were filter-sterilized then added to the media when it had cooled to 40 - 50°C before was dispensed into plates and culture bottles.

7.3.5 Infection and co cultivation

A. tumefaciens cultures were grown for 3 days in the dark at 28°C on YEP medium plates supplemented with 100 mg/l Spectinomycin and 100 mg/l Kanamycin. Two transformation procedures were evaluated.

Procedure 1: A single colony of Agrobacterium was inoculated in 5 ml of YEP medium supplemented 100 mg/l Spectinomycin and 100 mg/l Kanamycin in a 50 ml falcon tube and grown at 28°C overnight on rotary shaker at 200 rpm to an optical density of the culture measured at 0.8. The overnight grown cells were spinned at 6,000 rpm for 10 mins and the supernant was discarded. The pellet was washed in plain YEP medium and later resuspended in liquid infection medium supplemented with 100μM Acetosyringone. The cells were then poured on the leaf discs and petioles explants followed by agitation for 10 min at 200 rpm on a rotary shaker in dark at room temperature.

Procedure 2: A single colony of Agrobacterium was inoculated in 5 ml infection medium in a 50 ml falcon tube and grown at 28°C in the dark for 4 to 5 hours on rotary shaker at 200 rpm to an optical density of the culture measured ≈ 0.5. The cells were then poured on the leaf discs and petioles explants followed by agitation for 10 min at 200 rpm on a rotary shaker in dark at room temperature.

After infection in transformation 1 and 2, the leaf discs and petioles were blot-dried on sterile filter papers then cultured on co cultivation medium. Plates were incubated in

the dark at 28°C for 3 days. The co-cultivated explants were rinsed several times with liquid resting medium containing 500 mg/l cefotaxime to eliminate excess bacteria, blot dried with sterile filter papers and transferred to solid resting medium containing 250 mg/l cefotaxime for callus induction under a 16 hr light/ 8 hr dark cycle at 26 ± 2°C.

Embryo response (%) was measured as the number of co cultivated leaf discs and petioles that had formed embryogenic callus at their surface after 4 to 7 days on resting media, compared with the total number plated. Embryogenic callus that formed was transferred to a medium which favoured the formation and proliferation of somatic embryos after 2 weeks. All embryos, whether responding or not, were transferred to selection medium.

7.3.6 Selection and Regeneration

After 4 weeks, somatic embryos were subcultured onto selection medium consisting of MS with 100 mg/l spectinomycin, 100 mg/l Kanamycin and 100 mg/l cefotaxime. Regenerated shoots (1.0 - 2 cm) were detached and transferred to a rooting medium of half strength MS with 0.3 mg/l IBA, with 100 mg/l spectinomycin and 100 mg/l Kanamycin.

7.3.7 Histochemical Analysis of Transient and stable *gus* expression

Histochemical staining of leaves was performed according to the method of Jefferson et al., (1987). Leaves of Kanamycin resistant shoots were excised and placed in fixative (10 Mm MES, 0.3 M mannitol, 100 mM and 0.3 % formaldehyde pH 5.6) on

ice for 30 min, followed by washing in 50 mM NaH2PO4, pH 7.0. The samples were stained overnight at 37°C in 50 mM NaH2PO4, 0.5 % (v/v) Triton X-100 and 1Mm 5-bromo-3-chloro-3-indoly-b-D-glucuronide cyclohexamide sodium salt (X-gluc) dissolved in dimethyl-formamide . Leaves were destained by repeated washing with 70 % ethanol and scored for GUS. The dark blue color observed in shoot tissues, was used as the indication of GUS activity.

Shoots with GUS activity were evaluated to determine the transient transformation efficiency. The percentages of shoots with GUS positive was defined as the numbers of GUS positive shoots per total numbers of evaluated shoots x 100%. From all GUS positive shoots, the frequency (%) of each shoot organ, i.e. shoot tip, leaf and stem, showing GUS activity was also calculated

7.3.8 Statistical Analysis

The data was analyzed using Excel. Each treatment consisted of at least two plates each with 8 explants and was replicated thrice. Frequency of GUS activity was calculated as number of plants showing expression to the total number of plants stained and is expressed as percentage. Data on the number of plants showing transient GUS expression was calculated.

7.4 Results

7.4.1 Sterilization

Exposure time of 20 min in 20% sodium hypochlorite as shown in section 4.4 1, table 4.2 gave a mean of 80 % sterilization on all accessions after repeating the experiment 3 times whereby each accessions had 20 explants hence 60 in total.

Table 7.1 Embryo sterilization of 3 accession using 20 % NaOCL for 20 min.

Accession	No of individuals tested	No of survived individual	% survival
JCO4	60	45	75
JE4	60	48	80
JN1	60	51	85
		Mean	80

7.4.2 Co cultivation

Infected leaf discs and petioles explants grown on MS supplemented with1.5 mg/l BAP, 0.6 mg/l KIN, 0.5 mg/l IAA and 0.1 mg/l TDZ showed some swellings at the edges after three days of co cultivation. Some minimal necrosis was observed on the explants in general due to the effect of co cultivation with Agrobacterium but when the explants were transferred to new media they recovered well.

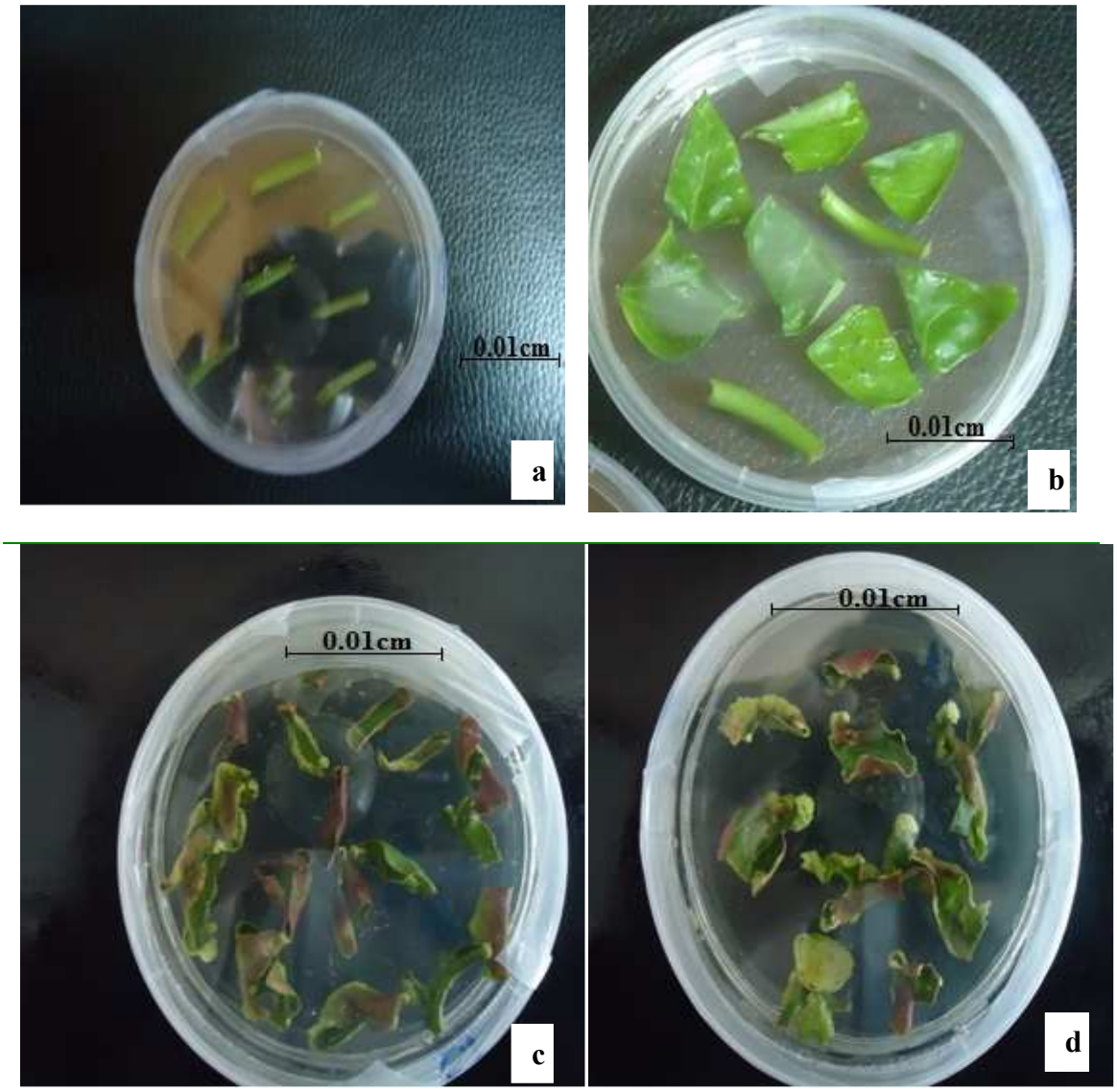


Figure 7.2: Transformation of petioles and leaf discs explants, a) Infected petiole explants b) infected leaf discs, c) procedure1 JCO4 d) procedure 2 JCO4.

7.4.3 Resting stage

When the infected explants were transferred to the resting media embryogenic calli were formed on all accessions at varying rates depending on the transformation

procedure. Each procedure was repeated 3 times whereby each accession had 10 explants hence a total of 30.

Transformation procedure 2 had more embryogenic calli formation compared to procedure 1 as shown in table 7.2. Procedure 2 gave callus induction of 85%, 90% and 95 % in accession JCO4, JE4 and JN1 respectively. The colour of the lower part of the leaf explants was purple in all leaf explants due to *Agrobacterium* infection but in petiole it remained green as shown in figure 7.3.

Table 7.2 Callus induction on transformed accessions

Accession	Experiment	No of individuals tested	% callus induction
JCO4	Transformation procedure 1	30	55
JCO4	Transformation procedure 2	30	85
JE4	Transformation procedure 1	30	60
JE4	Transformation procedure 2	30	90
JN1	Transformation procedure 1	30	50
JN1	Transformation procedure 2	30	95
Mean			72.5

After embryogenic calli were transferred to selection media, petioles were observed to respond better than leaf discs explants with more and healthy calli. At this stage the colour of the lower surface of the leaf had less purple colour than during the resting stage as shown in figure 7.4.

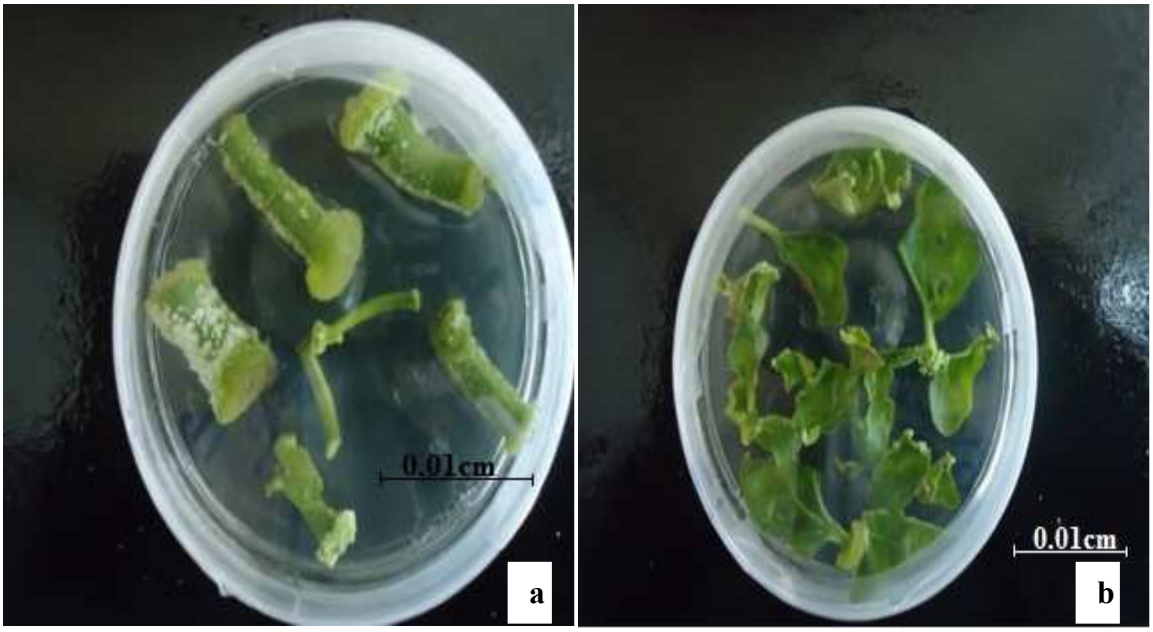


Figure 7.3: Callus induction on both petioles and leaf discs a) petiole callus b) leaf callus

7.4.4 Embryo development

During selection, some calli became brown and senesced while majority were embryogenic (Figure 7.5 a and b). Globular, heart and torpedo embryo development stages were observed.

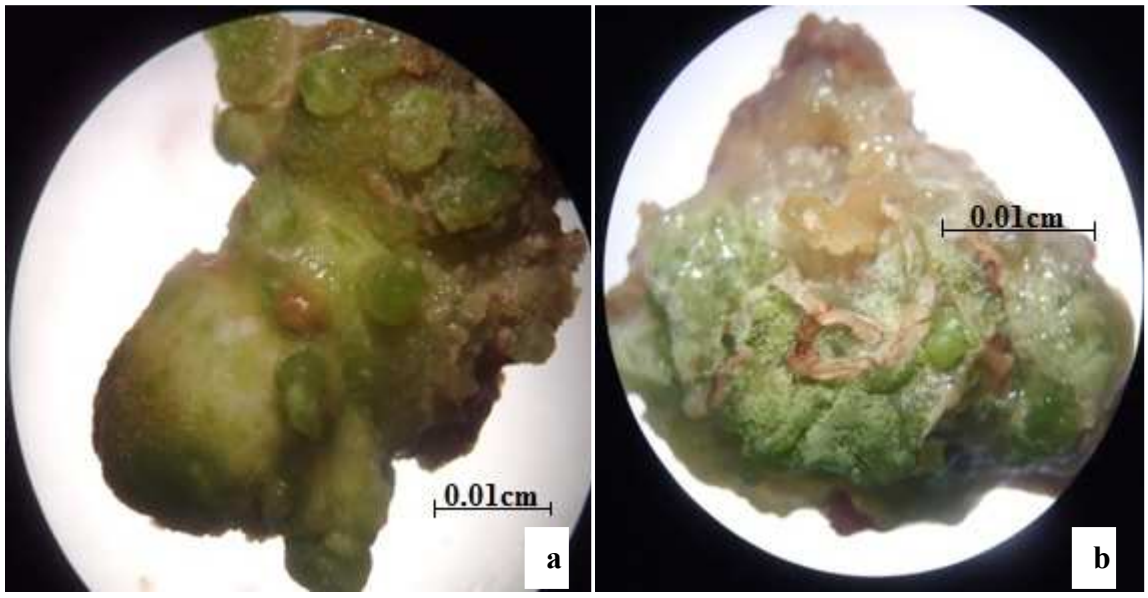


Figure 7:4 Embryogenic cultures of Jatropha a) Embryogenic callus from petiole b) Embryogenic callus from a leaf.

7.4.5 Embryo Germination

On subculturing of embryogenic calli on selection media, the transgenic ones germinated to form buds (Figure 7.6 a) while non transgenic ones started senescing (Figure 7.6 b). The transgenic buds developed into shoots as shown in figure 7.6 c and d. Although petioles were observed to form more embryogenic callus, high embryo development was observed in leaf discs. The generated shoots showed typical Jatropha morphology. After subsequent sub culturing on the same medium, more shoots proliferation and elongation was achieved. Transgenic plantlets which were Kanamycin and Spectinomycin resistant were obtained after 6 weeks of culture on selection media (Figures 7.6 e and f).

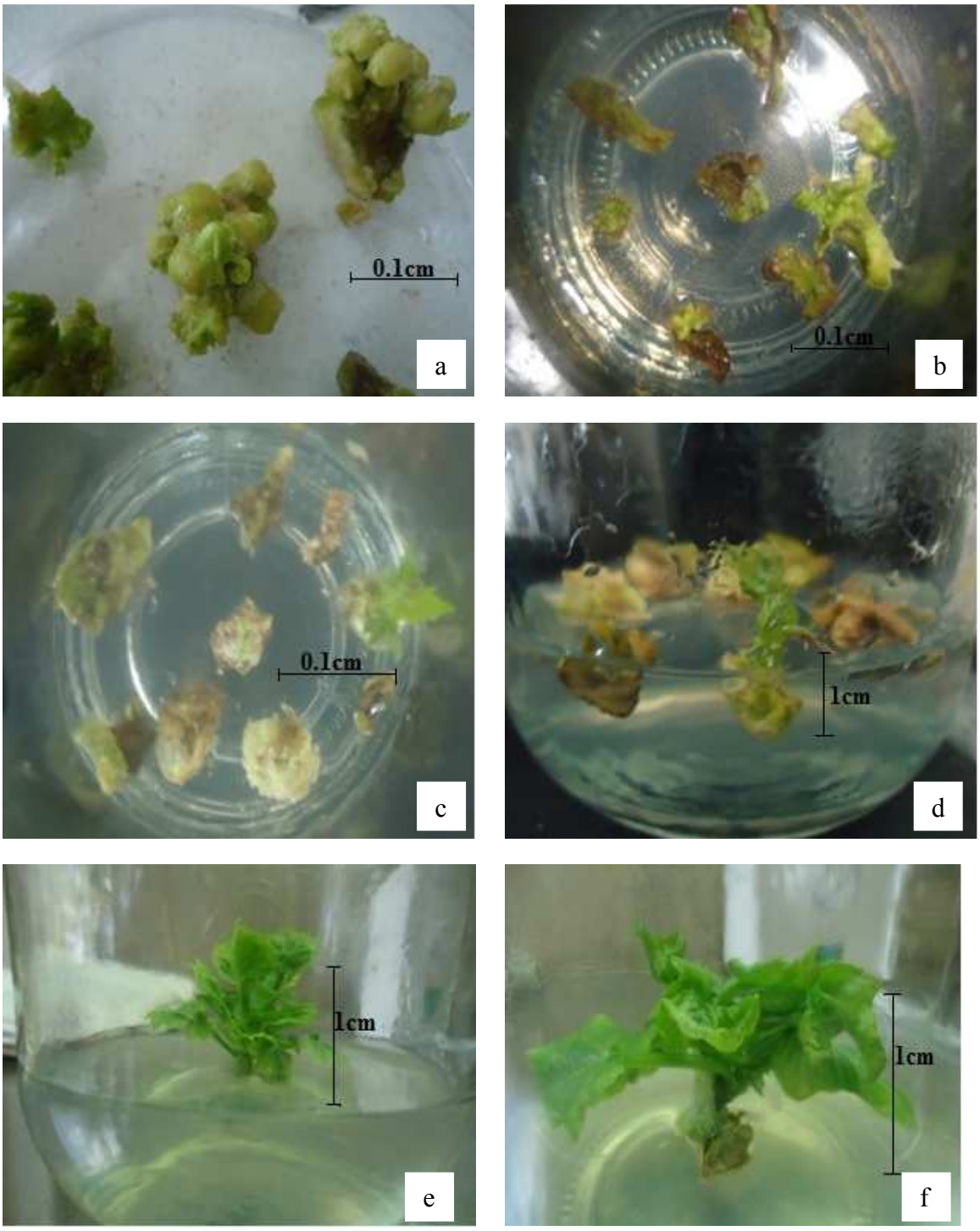


Figure 7:5 Development and selection of transformants a) Embryo germination, b) embryo senesces c) shoot development d) shoot elongation e) and f) selected of putative transformants

7.4.6 GUS assay

Histochemical GUS assays showed that *aadA* was expressed in resistant shoots by a blue colour signal (Figure 7.7a and b) whereas no blue GUS signal was observed in non transformed shoots (Figure 7.7c). In general procedure 2 gave more GUS positive results across all the accession compared to procedure 1. Procedure 2 gave 50%, 60% and 58.3% GUS positive shoots in accession JCO4, JE4 and JN1 respectively, while procedure 1 gave 40%, 33.3% and 25 % GUS positive plantlets in accessions JCO4, JE4 and JN1 respectively. A mean transformation efficiency of 44.43 % was observed.

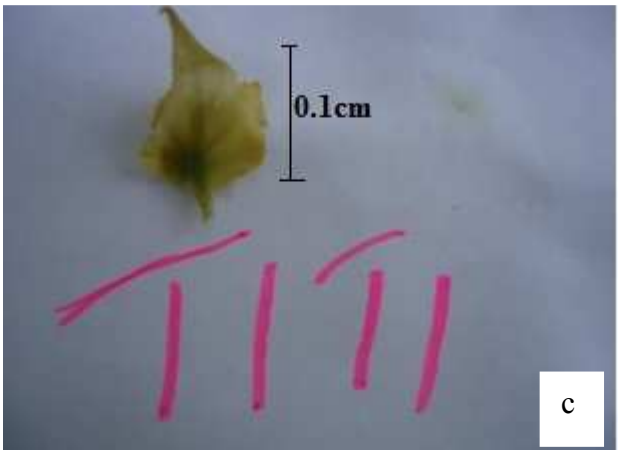


Figure 7.6: Transgenic plants showing histochemical GUS staining a, b and c.

Table 7.3 Transformation efficiency in six Agrobacterium transformation procedures

Accession	Experiment	No of individuals regenerated	GUS +ve	% GUS +ve
JCO4	Transformation procedure 1	5	2	40
JCO4	Transformation procedure 2	8	4	50
JE4	Transformation procedure 1	6	2	33.3
JE4	Transformation procedure 2	10	6	60
JN1	Transformation procedure 1	4	1	25
JN1	Transformation procedure 2	12	7	58.3
Mean				44.43

7.5 Discussion

Leaves were more susceptible to *Agrobacterium* infection due to their increased surface they later rejuvenated and produced more shoots hence preferred explants in Jatropha transformation (Meiru *et al.,* 2008; Li *et al.,* 2006). The callus induction, development, growth and shoot formation varied from one accession to another as a result of different genetic make up as shown in RAPD analysis whereby accessions from Coast (JCO4), Eastern (JE4) and Nairobi (JN1) clustered separately.

Sterilization procedure used during *in vitro* protocol development gave good mean survival (80%) for seed embryos used in transformation. To define whether our regeneration protocol was transformable we tested 3 out of 4 accessions that were successfully regenerated in *in vitro* conditions. The three accessions gave a mean of 72.5 % callus induction and 44.43 % GUS positive shoot. The two selectable antibiotics used for selections were 100mg/l Kanamycin and 100mg/l spectinomycin. The results showed that, they both completely blocked shoot regeneration from untransformed calli and led to shoot regeneration from transformed calli. Incase untransformed calli grew up to shoots, the shoots were killed. The suppressed callus turned brown while the other calli remained greenish to white green until it regenerated shoots.

Explants were incubated with the Agrobacterium for two different time intervals. When the two transformation procedures were compared the second procedure gave better results than the first one in all stages of growth and across all accessions. It was

observed that transient expression of GUS increased with the incubation time according to Li *et al.,* 2006. However the petioles had high rate of callus induction compared to leaf discs although leaf discs showed higher shoot regeneration. Therefore use of Kanamycin and spectinomycin for selection in this study was successful.

Suppressing of Agrobacterium after co- cultivation in this study was done using cefotaxime which did not inhibit both callus and shoot regeneration rate of both leaf discs and petioles (Li *et al.,* 2006). Another important factor which potentially influenced transformation is the Agrobacterium strains which vary according to the susceptibility of plant species. EHA101 bacterial strain harboring the binary vector pTF102, which contain the *bar* selectable marker gene and the GUS reporter gene produced different transient blue GUS spots where several blue spots were detected per leaf. The results indicate that EHA101 strain was efficient in producing both resistant calli allowing shoot formation. Agrobacterium EHA101was therefore selected for further Jatropha curcas transformation experiments. Histochemical GUS assays showed that *aad*A was expressed in resistant shoots whereas no blue GUS signal was observed in non transformed shoots.

7.6 Conclusion

Moderately efficient genetic transformation and plant regeneration methods were established for Jatropha using both leaf discs and petioles. Present findings on Jatropha indicated that leaf discs explants could serve as the best transformation

material. The developed *in vitro* regeneration protocol is amenable to *Agro bacterium* mediated transformation.

7.7 Recommendations

PCR and Southern hybridization should be done to confirm the transformation with PCR primers specific to bar gene. Further work on transformation should be carried out to transform Jatropha with genes for desirable traits.

CHAPTER EIGHT

8.0 GENERAL DISCUSSION, CONCLUSION AND RECOMMENDATIONS

Survey results in this study show that Jatropha has attracted a lot of interest which has triggered investments and expansion of cultivation areas in several parts of the country. Popular claims on Jatropha drought tolerance, low nutrient requirement, pest and disease resistance and high yields (Openshaw, 2000) were observed to have triggered Jatropha hype in most of the farmers. Most of these claims were not in agreement with the finding of this study. Jatropha was noted to be affected by several pests and diseases among other production constraints (Table 3.7). Due to the intensive red beetle infections on the experimental plot at JKUAT farm which was a big challenge to collect data on the plant morphology. This resulted to dormancy of the plant and delayed yielding time. Diseases like root rot led to death and falling of some plants. Better growth and development in Green Africa Foundation, Kitui where agronomic and cultural practices were properly carried out showed that Jatropha just like any other crop needs good management if its maximum benefits have to been attained. Existing knowledge on the species management among farmers should be integrated and disseminated to farmers and NGO's who are involved in Jatropha production.

This study shows that monoculture cropping was more prone to diseases and pest compared to intercropping. Although farmers were excited with the new crop they lack general knowledge on best practices, pest and disease management and economic

viability. Some of these problems could be overcome by intercropping Jatropha with other crops. Integrating Jatropha into existing smallholder agro forestry systems, such as a hedge can form a robust and sustainable base for Jatropha domestication and expansion (Achten *et al.,* 2007). This should be prioritized based on need and compatibility with the existing farming system.

The distribution and management of Jatropha was also investigated and documented. Several accessions of Jatropha both landrace and wild type were reported to be grown in Kenya. A wide range of agronomic and cultural practices were reported that can be assessed for better Jatropha production (Table 3.5). The number of accessions and diverse use of local names of Jatropha by different communities in this study reflected a wide distribution and use of Jatropha in Kenya. This study shows that Jatropha is found growing in the Coast, Eastern, Rift Valley, Central, Nairobi and Western parts of Kenya which are characterized by savannah and semi arid climate. Earlier reports by Muok *et al.,* (2008) show that there may be more distributions in Kenya like Naivasha and Marakwet. This was lacking in the KEFRI mapping, MOA and Eastern Africa Herbarium records (personal observations) hence need for better record keeping. Among the areas studied, Eastern region had a higher diversity. This was confirmed by the fact that out of 96 accessions sampled 41 (42.71%) were from Eastern. This diversity could be as a result of favourable ecological needs of Jatropha.

Namanga and Meru Jatropha accessions were reported to be over 50 years old which in agreement with earlier reports that the species was introduced into Kenya in 16th century (Muok and Kallback, 2008). At the age of 50 the species was still bearing

fruits which correspond with Heller, 1996 that it can yield up to 50 years. Meru and Magadi accessions were observed to have big seeds, resistance to pests and diseases and appeared well adapted to the environment. These accessions could be used in selection and breeding programs or a source of desirable genes for genetic improvement of various traits of Jatropha especially resistance to pests and disease.

Jatropha occurrence and distribution gives important information on availability of quality planting materials and ecological requirements for production. In its natural area of distribution Jatropha is most abundant in tropical savannah while it is not common in semi arid areas and it's totally absent in arid areas (Maes *et al.,* 2009). In this study Jatropha in Kenya was found to be more abundant in tropical savannah compared to semi arid areas.

Understanding the diversity and distribution of a given gene pool is a foundation for its genetic improvement. For genetic resources to be of valuable use they need to be effectively conserved and properly utilized (Zhang *et al.,* 1999). Effective conservation requires an available and reliable genetic diversity (Bekele *et al.,* 2005). Genetic resources are threatened with loss through genetic erosion due to environmental, social, political and economic challenges in the country. Thus genetic conservation and improvement based on the quality of selected materials should be encouraged with an aim of preventing erosion. Narrow gene pool could be due to its ability to be vegetatively propagated within a short time (Henning, 2007; Lengkeek, 2007). Majority of the regions surveyed demonstrate extremely low plant densities whereby genetic diversity due to pollen transfer is limited (Dawson, 2009).

In this study, the genetic diversity of Jatropha accessions collected from six major Jatropha growing regions (Coast, Eastern, Rift Valley, Central, Nairobi and Western) of Kenya were studied using molecular markers and morphological traits. Use of morphological and molecular tools in Jatropha studies played an important role of identification and characterization. Six morphological traits used to evaluate 11 Jatropha accessions showed that there is minimal morphological diversity in this species which concurs with the findings of Dehgan, (1979). Both qualitative and quantitative traits are recommended since quantitative traits are more sensitive to environmental influences and the growth stage of the plant. Use of RAPD markers for 69 Jatropha accessions revealed a substantial genetic diversity in Jatropha. Accessions from Eastern presented a higher variation compared to other accessions. Cluster analysis result compared to PCA analysis gave a clear and more informative display of the relative positions of the accessions. However small these accessions are, they serve as source for desirable genes for the genetic improvement of this crop.

When morphological and molecular characterization analyses were compared there was no clear similarity on the results. In morphological analysis Rift Valley accessions represented by Namanga were noted to be spread across the Coast and Eastern regions while in molecular analysis they had only spread to Nairobi region. These shows that molecular characterization gives refined results are described by Basha and Sujatha, 2007 compared to morphological. However combinations of the two methods in studying genetic diversity in Jatropha should be combined while carrying out improvement programs as morphological data contain important adaptive characters.

Quantitative characters documented can be used in improvement as they are more sensitive to environmental influence and growth stage of the plant.

Genetic diversity based on RAPD markers was on average higher than morphological traits. This results show that when both tools are used they might not give matching results. Two reasons might have lead to low correlation between DNA markers and morphological traits according to Semagn, 2002. While DNA markers cover a larger portion of the genome including coding and non coding regions, morphological markers cover less. Morphological markers are more subjected to artificial selection than DNA markers. Mart'nez *et al.,* (2005) believed that analysing more morphological characters and DNA markers can improve the correlation between the two methods.

In addition the use of tissue culture and genetic engineering tools was also explored in this study. Efficient regeneration protocol via somatic embryogenesis was established on Jatropha accessions. Transformability of the species was successfully reported. These findings show possibilities of transforming Kenyan Jatropha with genes for desirable traits.

Development of an efficient *in vitro* regeneration protocol opens an opportunity for mass propagation of elite varieties of Jatropha compared to the current use of conventional methods. This may be adopted for future research on transformation studies to improve this species. Use of somatic embryogenesis in plant regeneration offers advantages over shoot organogenesis in that multiplication rate can be faster

(Ammirato, 1983). Thidiazuron has also been used to stimulate somatic embryogenesis (Neuman *et al.,* 1988, 1992) and organogenesis (Fiola *et al.,* 1990; Bates *et al.,* 1992) in Walnut.

Conclusion

Jatropha is a new crop in Kenya which requires substantial research before it can be exploited as a commercial crop. Integration of Jatropha crop management and varieties improvement can improve its production and utilization. Use of both molecular and morphological approaches gives a better overview of the genetic diversity of Jatropha in Kenya. There are no improved Jatropha varieties and no tissue culture planting material is available to Kenyan famers.

Recommendations

Studies should be carried out to determine the best agronomic and cultural practices, pest and disease management in Jatropha cultivation. This could help improve production and utilization of the crop. Production of quality and quantity planting material should be done. Tapping of other products from Jatropha such as traditional medicine from its leaves, latex, fruits coats, seed cake and shells, fertilizers, insecticides and soap production should be encouraged. These could generate more income to the farmers other than biodiesel only. Breeding programmes on the black listed accessions on the Kenyan germplasm should be done. Proper biofuel production policies in Kenya should be put in place especially concerning land use and biodiversity conservation. This could solve the many issues facing Jatropha

production, processing and marketing. Genetic improvement studies should be done to improve the crop production.

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APPENDICE

Appendix I: Questionnaire

<table>
<tr><td colspan="3">Collection no.</td><td colspan="2">Collection date</td></tr>
<tr><td colspan="3">Local name</td><td colspan="2">Province</td></tr>
<tr><td colspan="3">District</td><td colspan="2">Division</td></tr>
<tr><td colspan="3">Locality</td><td colspan="2">Farmers name</td></tr>
<tr><td colspan="3">Farm size</td><td colspan="2">Source of collection</td></tr>
<tr><td colspan="3">Genetic status</td><td colspan="2"></td></tr>
<tr><td colspan="2">Habitat type</td><td colspan="3">Habitat description (flat, slope)</td></tr>
<tr><td>Intercropped</td><td colspan="4">Height Age branching level
Leaf colour Stem colour</td></tr>
<tr><td rowspan="2">Local frequency</td><td rowspan="2">Flower phenology</td><td rowspan="2">Fruit phenology</td><td>Planting time</td></tr>
<tr><td>Planting material</td></tr>
<tr><td colspan="2">Harvesting time</td><td colspan="3">Origin Seed: Local Elsewhere</td></tr>
<tr><td colspan="2">Spacing</td><td colspan="3">Yields (amount and frequency)</td></tr>
<tr><td colspan="2" rowspan="3">Production constraints</td><td colspan="3">- Diseases and pests</td></tr>
<tr><td colspan="3">- Availability of water</td></tr>
<tr><td colspan="3">- Threats</td></tr>
<tr><td colspan="2" rowspan="2">Field management</td><td colspan="3">- Cultural practices</td></tr>
<tr><td colspan="3">- Agronomic practices</td></tr>
<tr><td colspan="2" rowspan="2">Local uses</td><td colspan="3">Drainage</td></tr>
<tr><td colspan="3">Topography</td></tr>
<tr><td colspan="2">Site description</td><td colspan="3">Soil texture</td></tr>
<tr><td colspan="2">Soil colour</td><td colspan="3">Stoniness</td></tr>
<tr><td colspan="2">Number of plants sampled</td><td colspan="3">Number of plants found</td></tr>
<tr><td colspan="2">Area sampled</td><td colspan="3">% of population seeding</td></tr>
<tr><td colspan="2">Material collected</td><td colspan="3" rowspan="2">Colour of collected material: Seed
Fruit

Cuttings</td></tr>
<tr><td colspan="2">Collection material
Method of collection</td></tr>
</table>

Appendix II: Jatropha collection from different counties of Kenya

Code	Province	District	Farm name	*Origin	**Sample	Habitat found
JE1	Eastern	Kitui	Kitui (G.A.F)	magadi	Seeds	Farm
JE2	Eastern	Kitui	Kitui (G.A.F)	China	Cuttings	Nurseries
JE3	Eastern	Kitui	Kitui (G.A.F)	Philippine	Seedling	Nurseries
JE4	Eastern	Kitui	Kitui (V.F)	unknown	Seedling	Farm
JE5	Eastern	Kitui	Katumo Home	unknown	Seed /cutting	Fence
JE6	Eastern	Kitui	Kyanika market	unknown	Seed /cutting	Market
JE7	Eastern	Kitui	Mutukya Market	unknown	Seed /cutting	Market
JE8	Eastern	Kitui	Mutukya river	unknown	Seed /cutting	River
JE9	Eastern	Kitui	Mutukya banks	unknown	Seed /cutting	River
JE10	Eastern	Kitui	Mutukya roadside	unknown	Seed /cutting	Bush
JE11	Eastern	Kitui	Mutukya 7	unknown	Seed /cutting	Farm
JE12	Eastern	Kitui	Malaani	unknown	Seed /cutting	Fence
JE13	Eastern	Kitui	Mutwei river	unknown	Seed /cutting	River
JE14	Eastern	Kitui	Mutwei home	unknown	Seed /cutting	Fence
JE15	Eastern	Kibwezi	Kibwezi Kikumb	School	Seedling	Fence
JE16	Eastern	Kibwezi	Kibwezi Kilo Fa	NGO	Seedling	Farm
JE17	Eastern	Kibwezi	Kibwezi Kefri	Meru	Seedling	Nurseries
JE18	Eastern	Kibwezi	Kibwezi Kefri	Magadi	Seedling	Nurseries
JE19	Eastern	Kibwezi	Kibwezi Kefri	Uganda	Seedling	Nurseries
JE20	Eastern	Kibwezi	Mbitini	unknown	Seeds	Fence
JE21	Eastern	Kibwezi	Kyulu hills	unknown	Seed	Bush
JE22	Eastern	Kibwezi	Matiliku Kasoo	Malindi	Seed	Fence
JE23	Eastern	Kibwezi	Matiliku Mwau	malindi	Seed	Fence
JE24	Eastern	Tharaka	Mtombo	unknown	Seed	Market
JE25	Eastern	Tharaka	Tharaka 4	unknown	Seed	Fence
JE26	Eastern	Tharaka	Marimati	unknown	Seed	Market
JE27	Eastern	Tharaka	Tharaka 5	unknown	Seed	Fence
JE28	Eastern	Tharaka	Tharaka	unknown	Seedlings	Fence
JE29	Eastern	Meru	Muriki	Meru	Seed /cutting	Fence
JE30	Eastern	Meru	Kathia	Meru	Seed /cutting	Fence
JE31	Eastern	Meru	Mbairu	Meru	Seed /cutting	Fence
JE32	Eastern	Meru	Geoffrey	Meru	Seed /cutting	Fence
JE33	Eastern	Meru	Mukethi	Meru	Seed /cutting	Fence
JE34	Eastern	Meru	Kimenju	Meru	Seed /cutting	Fence
JE35	Eastern	Meru	Kanaire	Meru	Seed /cutting	Fence
JE36	Eastern	Meru	Nchiru Market	Meru	Seed /cutting	Market
JE37	Eastern	Meru	Elijah	Meru	Seed /cutting	Fence
JE38	Eastern	Meru	Stanley	Meru	Seed /cutting	Fence
JE39	Eastern	Meru	Kaja July	Meru	Seed /cutting	Fence
JE40	Eastern	Meru	MKU	Meru	Seed /cutting	Farm
JE41	Eastern	Meru	Miriti Pat	Meru	Seed /cutting	Fence
JE42	Eastern	Meru	Muturo Dav	Meru	Seed /cutting	Fence
JRV1	Rift valley	Namanga	Margaret	unknown	Seed	Fence
JRV2	Rift valley	Namanga	Fredrick	unknown	Seed	Farm

Apendix 11 cont'

JRV3	Rift valley	Namanga	Muchiri	unknown	Seed	Farm
JRV4	Rift valley	Namanga	Abdi	unknown	Seed	Fence
JRV5	Rift valley	Namanga	A.P	Police st.	Seed	Reserve
JRV6	Rift valley	Namanga	G.P	British	Seed	Reserve
JRV7	Rift valley	Magadi	Le papa	Unknown	Seed	Fence
JRV8	Rift valley	Magadi	Ntore	Unknown	Seed	Fence
JRV9	Rift valley	Magadi	Kasio	Unknown	Seed	Fence
JRV10	Rift valley	Magadi	Mwanzu	Unknown	Seed	Fence
JRV11	Rift valley	Magadi	Parmul	Unknown	Seed	Fence
JRV12	Rift valley	Magadi	Lemunke	Unknown	Seed	Fence
JRV13	Rift valley	Magadi	Albert	Unknown	Seed	Fence
JRV14	Rift valley	Magadi	Lesion	Unknown	Seed	Fence
JRV15	Rift valley	Magadi	Kolei	Unknown	Seed	Fence
JRV16	Rift valley	Magadi	KARI Magadi	Unknown	cutting	Fence
JN1	Nairobi	Nairobi	National Museum	Kisumu	Seed /cutting	Reserve
JN2	Nairobi	Nairobi	National Museum	Coast	Seedling	Reserve
JN3	Nairobi	Nairobi	National Museum	Coast	Seedling	Reserve
JN4	Nairobi	Nairobi	National Museum	Coast	Seedling	Reserve
JN5	Nairobi	Nairobi	National Museum	Tharaka	Seedling	Reserve
JN6	Nairobi	Nairobi	National Museum	Tharaka	Seedling	Reserve
JN7	Nairobi	Nairobi	National Museum	Tharaka	Seedling	Reserve
JC1	Central	Thika	IEET Farm	Magadi	Leaves	Farm
JC2	Central	Thika	IEET Farm	Magadi	Leaves	Farm
JC3	Central	Thika	IEET Farm	Magadi	Leaves	Farm
JC4	Central	Thika	IEET Farm	Magadi	Leaves	Farm
JC5	Central	Thika	IEET Farm	Magadi	Leaves	Farm
JC6	Central	Thika	IEET Offices	Magadi	Leaves	Farm
JC7	Central	Thika	IEET Offices	Magadi	Leaves	Farm
JC8	Central	Thika	IEET Offices	Magadi	Leaves	Farm
JC9	Central	Thika	IEET Offices	Magadi	Leaves	Farm
JC10	Central	Thika	IEET Offices	Magadi	Leaves	Farm
JC11	Central	Thika	BEED Farm	unknown	Leaves	Farm
JC12	Central	Thika	BEED Farm	unknown	Leaves	Farm
JC13	Central	Thika	BEED Farm	unknown	Leaves	Farm
JC14	Central	Thika	BEED Farm	unknown	Leaves	Farm
JNY1	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY2	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY3	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY4	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY5	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY6	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY7	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY8	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm
JNY9	Nyanza	Kisumu	Kisumu market	unknown	Seedlings/seeds	Farm
JNY10	Nyanza	Kisumu	Kisumu market	unknown	Seeds/seedlings	Farm

Apendix 11 cont'

JCO1	Coast	Kilifi	Kefri Arabuko	unknown	Seed /cutting	Farm
JCO2	Coast	Kilifi	Mashafa Home	unknown	Seed /cutting	Fence
JCO3	Coast	Samburu	Kinango Bati	unknown	Seeds/seedlings	Fence
JCO4	Coast	Malindi	Malindi Markebu	NGO	Seed /cutting	Farm
JCO5	Coast	Kwale	Kinango Vuma	unknown	Seeds	Fence
JCO6	Coast	Samburu	Msabweni Tiomi	Tanzania	Seeds	Farm
JCO7	Coast	Kwale	Gede Museum	Tanzania	Seedlings	Reserve

G.A.F - (Green Africa Foundation) **V.A** - (Vanilla Foundation) A.P- Administrative police post] G.P – General police post

***Origin** - source of planting material ****Sample** – plant or plant part collected

Habitat – the ecology in which the plant was growing in.

Appendix III: Murashige and Skoog (1962) plant culture medium

Essential element	Constituents	Concentration in stock solution (g/l)
Macronutrients[a]	KNO_3	38.0
	NH_4NO_3	33.0
	$CaCl_2.2H_2O$	8.8
	$MgSO_4.7H_2O$	7.4
Micronutrients[a]	KH_2PO_4	3.4
	H_3BO_3	0.124
	$MnSO_4.4H_2O$	0.446
	$ZnSO_4.7H_2O$	0.172
	$Na_2MoO_4.2H_2O$	50mg
	$CuSO_45H_2.O$	5mg
	$CoCL_2.5H_2O$ KI	5mg 16.6mg
Organic supplement[b]	Myo-inositol	2g
	Nicotinic Acid B3	10mg
	Pyridoxine-HCl	10mg
	Glycine	10mg
	Thiamine-HCl	0.1mg
Iron source[c]	$Na_2EDTA.2H_2O$	745mg
	$FeSO_4.7H_2O$	557mg
Carbon source[d]	Sucrose	Added as solid

Appendix IV: DNA Extraction Reagents

CTAB buffer (1 l)

100 ml of 1 M Tris, pH 8.0

280 ml of 5 M NaCl

40 ml of 0.5 M EDTA

20 g of CTAB, 20g PVP (Polyvinylpyrrolidone)

Bring the total volume to 1 L with ddH_2O

Autoclave at 121°c 20 min

1 M Tris, pH 8.0(1 L)

121.1 g Tris

Dissolve in 700 ml ddH_2O

Bring pH to 8.0 by adding concentrated HCl

Bring the total volume to 1 L with ddH_2O

0.5 M EDTA pH 8.0 (1 L)

186.12 g of EDTA

Add about 700 ml ddH2O

Add about 20 g of NaOH pellets

Adjust pH to 8.0 by adding few NaOH pellets

Bring the total volume to 1 L with ddH_2O

TE buffer (1 L)

10 mM 10 ml of 1 M Tris, pH 8.0

1 mM 2 ml of 0.5 M EDTA

Bring the total volume to 1 L with ddH_2O

5 M NaCl (1 L)

292.2 g of NaCl, 700 ml ddH2O

Dissolve and bring to 1 L

70% ethanol (100ml)

Mix 70 ml ethanol with 30ml of previously autoclaved distilled water

Chloroform – isoamyl alcohol (24:1) (100ml)

In a clean dark bottle, mix 96ml of chloroform with 4ml of isoamyl alcohol

5 x TBE stock solution (5 litres)

270 g Tris

Add 4000 ml distilled water

Add 138 g boric acid and dissolve

Add 100 ml 0.5 EDTA (pH 8.0)

Bring the total volume to5 L with ddH_2O

6 X agarose gel loading dye (100ml)

50 g glycerol

Add 250 mg bromophenol blue

Add 250mg xylene cyanol

Bring the total volume to 100ml with ddH_2O

10 mg/l ethidium bromide stock solution

0.1g ethidium bromide

Add 10 ml distilled water and dissolve